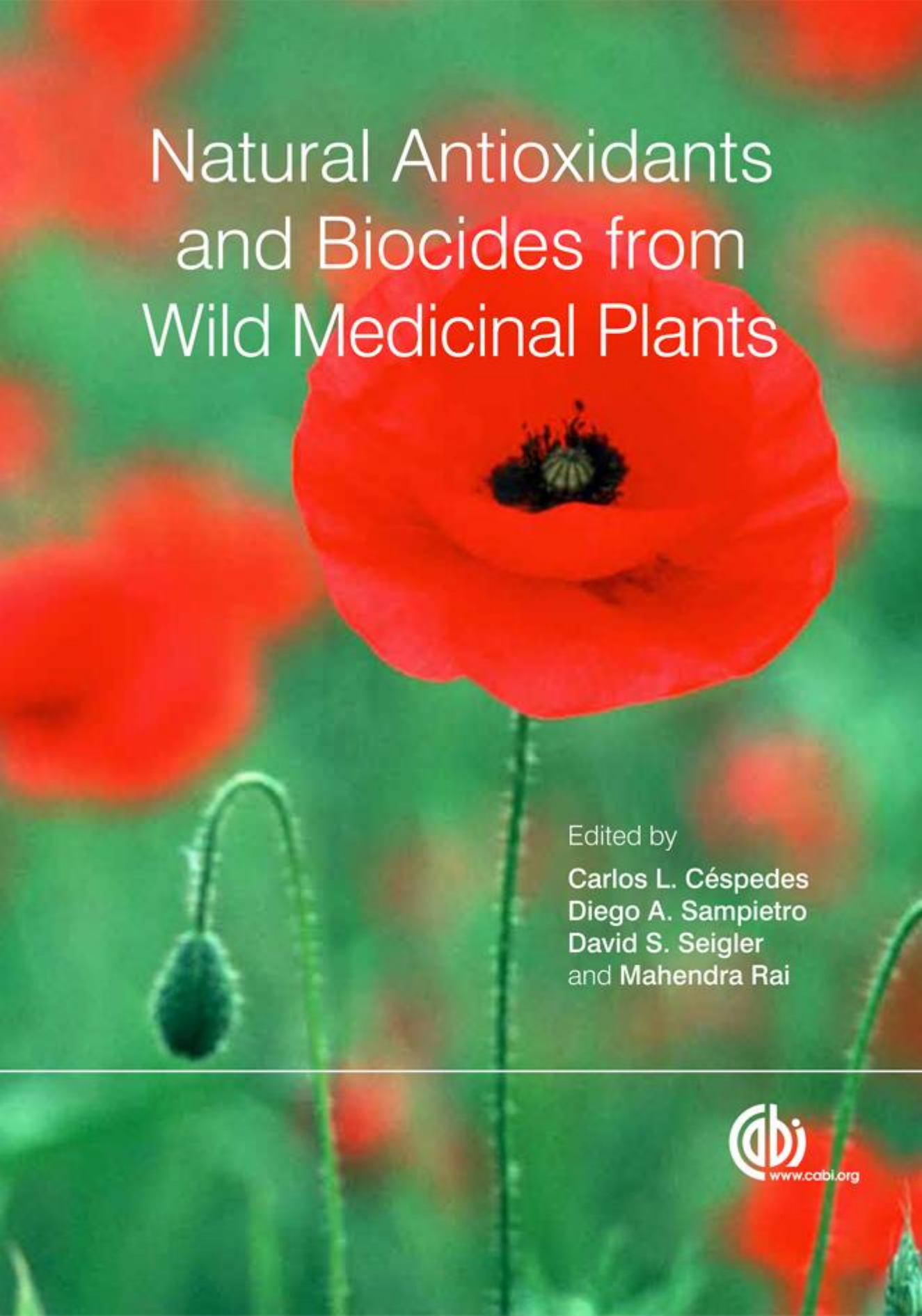
Natural Antioxidants and Biocides from Wild Medicinal Plants
Edited by
Carlos L. Céspedes
Diego A. Sampietro
David S. Seigler
and Mahendra Rai
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Dr Céspedes is Full Professor and Senior Researcher at the Basic Sciences Department, University of Bío-Bío (Chillán, Chile). He graduated from Pontificia Catholic University (Chile) as Teacher of Science (Chemistry mention) in 1982, then completed his Master of Science degree (1988) and PhD (1994) in Chemical Sciences at the University of Concepción (Chile). Following a postdoctoral position from 1996 to 1998 at the Chemistry Institute of the National Autonomous University of Mexico (UNAM), he remained at the university, working in natural products chemistry until 2006, investigating natural products, chemical interactions among plants and other organisms, and their applications. He has been invited for postdoctoral research stays in several universities including: the University of Urbana-Champaign, Illinois, by Prof. David Seigler; the University of California at Berkeley, by Prof. Isao Kubo; the University of Milan in Italy, by Prof. Daniele Passarella; the University of Cadiz in Spain, by Prof. Francisco A. Macias; and at the Chemistry Institute, University of Antioquia in Colombia, by Prof. Fernando Echeverri. Since 1990 he has been investigating secondary metabolites involved in plant–plant and plant–insect interactions from Latin American plants belonging to the Agavaceae, Asteraceae, Cactaceae, Celastraceae, Elaeocarpaceae, Euphorbiaceae, Leguminosae, Meliaceae and Zygophyllaceae families. He has co-published two books and participated as author/co-author on more than 82 scientific publications, including ten book chapters and more than 120 communications in international meetings.

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Dr David Seigler
Dr Seigler graduated from Southwestern Oklahoma State University (1961) where he majored in Chemistry. He earned his doctorate in Organic Chemistry in 1967 from the University of Oklahoma. He then completed postdoctoral work at the Northern Regional Laboratory of the United States Department of Agriculture in Peoria, Illinois, and at the University of Texas in Austin. Dr Seigler is currently an emeritus professor in the Department of Plant Biology at the University of Illinois at Urbana-Champaign, where he has been since 1970. During that time, he has authored/co-authored over 160 publications and has participated in several book projects. Seigler's research focus has been on the role of plant compounds in biological problems, such as plant–insect, plant–fungal and plant–herbivore interactions. Much of his work has involved cyanide and tannins from plants, and research on *Acacia* and *Passiflora* species. He has conducted research in several Latin American countries and received the Fulbright-Hays Lecturing Research Grant to teach at the University of Buenos Aires in Argentina. He has served as President of the Phytochemical Society of North America and the International Society of Chemical Ecology. In addition to his departmental work at the University of Illinois, Seigler serves as the Curator of the University of Illinois Herbarium (ILL), research associate with the Missouri Botanical Garden in St. Louis, and Affiliate Professional Scientist with the Illinois Center for Economic Entomology.

Dr Mahendra Rai
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Preface

The current book provides an up-to-date treatment of antioxidant and biocidal compounds mainly from Latin American plants. New antimicrobials, insecticides and antioxidants are compiled in a single source for the first time based on the research and knowledge of several internationally renowned research groups. The book is organized in three sections: Part I provides a general overview and perspectives on antioxidant, medicinal and biocidal plant compounds; Part II provides information on plant antioxidants isolated from a wide range of species; and Part III describes insecticidal, antimicrobial and other biocidal activities based on peptides, phytoecdysteroids, alkaloids, polyphenols, terpenoids and other allelochemicals. This book will be a helpful reference for students, researchers and teachers interested in allelochemistry as well as the antioxidant and medicinal properties of Latin American plants.

Carlos L. Céspedes
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1 Antioxidant and Biocidal Activities from Natural Sources: an Overview

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1.1 Introduction

Plant natural products derived from isoprenyl pyrophosphate, phenylalanine and the phenylpropanoid pathway are impressive in their chemical diversity and are the result of plant evolution that has selected for the acquisition of large repertoires of structural and defensive compounds, all derived from a phenylpropanoid backbone via the plant-specific shikimic acid pathway. These compounds are important for plant growth, development and responses to environmental stresses and thus can have a large impact on agricultural productivity. Although plant-based medicines containing phenylpropanoid-derived active components have long been used by humans, the benefits of specific flavonoids and other phenylpropanoid-derived compounds to human health and their potential for long-term health benefits have only been recognized more recently.

This overview brings together widely scattered and disparate literature concerning natural products from Latin-American plants; in particular studies based on screening of secondary metabolites are reviewed in the context of the development of new antioxidants and biocides.

Despite considerable progress towards characterizing compounds that inhibit growth of insect pests and weeds, evidence for an ecological role for these compounds is poor. Even if a broad range of biocides of sufficient promise is discovered, major hurdles must be overcome before the compounds can be commercially exploited.

A number of recent phytochemical studies have been directed to identifying biocides of botanical origin. Anthocyanins, diterpenes, triterpenes, sesquiterpene lactones, coumarins, flavonoids and phenylpropanoids have been isolated from, for example, the Anacardiaceae, Agavaceae, Asteraceae, Celastraceae, Elaeocarpaceae, Fabaceae, Leguminoseae, Loranthaceae, Meliaceae, Plantaginaceae, Rhamnaceae, Scrophulariaceae and Simaroubaceae families. Many of these natural compounds and some of their chemical derivatives possess antioxidant, antifungal, antibacterial, insecticidal, insect-growth-regulatory (IGR) or antifeedant activities. Much less is known about these and other plants from Latin America and the effects of their constituent compounds on bacteria, fungal and insect pests. In addition, a large proportion of these compounds also demonstrated antioxidant

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properties, as judged by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and other *in vitro* antioxidant assays. Our results indicate that many of the compounds from these families have excellent activity as antioxidants and have powerful effects on the metabolism of insects, bacteria and fungi. The naturally occurring compounds that we have isolated represent a valuable resource for both useful nutraceutical and biocide activities.

1.2 Antioxidants

Antioxidants are substances that delay the oxidation process by inhibiting polymerization chains initiated by free radicals and other subsequent oxidizing reactions (Halliwell, 1991, 1999). This concept is fundamental to food chemistry, in which synthetic antioxidants such as butylated hydroxy toluene (BHT) have long been used to preserve quality of food by protecting against oxidation-related deterioration. A growing body of literature points to the importance of natural antioxidants from a large number of plants that could be used to reduce oxidative damage, not only in foods but also in the human body. This might provide protection against chronic diseases, including cancer and neurodegenerative diseases, inflammation and cardiovascular disease. Adverse conditions within the environment, such as smog and UV radiation, in addition to diets rich in saturated fatty acids, increase oxidative damage in the body. Given this constant exposure to oxidants, antioxidants may be necessary to counteract chronic oxidative effects, thereby improving the quality of life (Roberts *et al.*, 2003).

The increasing interest in the measurement of the antioxidant activity of different plant samples is derived from the overwhelming evidence of the importance of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet -}$), alkoxyl ($RO^{\bullet -}$), hydroxyl ($HO^{\bullet -}$), and nitric oxide ($NO^{\bullet}$) radicals in ageing and chronic disease (Fernandes *et al.*, 2004). Several methods have been developed to measure the antioxidant activity in biological samples, including oxygen radical absorption capacity (ORAC), ferric reducing antioxidant power (FRAP), DPPH, crocin radical scavenging and inhibition of the formation of thiobarbituric acid reactive species (TBARS) (Taruscio *et al.*, 2004) as a more complete tool for antioxidant measurements (Schinella *et al.*, 2002; Prior *et al.*, 2005).

Berries from South America constitute a rich dietary source of phenolic antioxidant and bioactive properties (Céspedes *et al.*, 2010a; Schreckinger *et al.*, 2010a,b). The Chilean wild black-berry *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae), an edible black-coloured fruit that reaches its maximum ripeness between December and March, is widely consumed during these months in Central and South Chile and the western region of Argentina. We reported the alkaloid composition of the leaves of *A. chilensis* (Céspedes *et al.*, 1990, 1993, 1995; Silva *et al.*, 1997); other botanical characteristics were previously reported (Céspedes *et al.*, 1995, 2008, 2010a). This plant has enjoyed popularity as an ethnomedicine for many years and has been used as an anti-inflammatory agent, for kidney pains, stomach ulcers, diverse digestive ailments (tumours and ulcers), fever and cicatrization injuries. The berries have traditionally been consumed as a treatment for diarrhoea and dysentery. Araucanian people prepare a liquor known as 'tecu' by macerating the fruits in ethanol for use in religious rituals and consumption as a daily beverage (Muñoz-Pizarro, 1966).

Some recent studies indicate that extracts from the fruits of *A. chilensis* have good antioxidant properties (Pool-Zobel *et al.*, 1999) and cardioprotective activity (Céspedes *et al.*, 2008). Yet other studies report the composition of extract constituents (Escribano-Bailon *et al.*, 2006; Céspedes *et al.*, 2010a). The inhibitory activity of an anthocyanin-rich ethanol extract of this fruit against aldose reductase, adipogenesis and the inhibition of expression of lipopolysaccharide (LPS)-induced nitric oxide

synthase (iNOS)/NO and cyclooxygenase-2 (COX-2)/PGE pathways in RAW 264.7 macrophages has been reported (Dominguez *et al.*, 2010; Schreckinger *et al.*, 2010a). Subsequently, the effects of a similar extract on an ischaemic/reperfusion system, several antioxidant activities of that extract, and the relationship between total phenolic levels and the cardioprotective effect (Céspedes *et al.*, 2008, 2010a), the presence of 3-hydroxyindole (Céspedes *et al.*, 2009), and the anti-inflammatory activity against 12-0-tetra-decanoyl-phorbol-13-acetate (TPA), have also been introduced into the literature (Céspedes *et al.*, 2010b).

As a continuation of our general screening programme for anti-inflammatory activity of *A. chilensis* (Céspedes *et al.*, 2010b), these fruit extracts have been investigated further in the carrageenan-induced paw oedema inflammation mouse model. In these studies, the ethanol and acetone extracts, their fractions and subfractions, the occurrence of phenolic compounds (Céspedes *et al.*, 2009, 2010a), and the correlations of the phytochemical content and gastroprotective effects were examined.

In general, the compounds that occur in *A. chilensis* are similar or identical to the active components of many other anti-inflammatory plants. Many phenolic acids, anthocyanins and flavonoids have been shown to have inhibitory activity on nitric oxide synthesis, which is implicated in physiological and pathological processes as a contributor to chronic inflammation (Matsuda *et al.*, 2000; Odontuya *et al.*, 2005). These findings substantiate that anthocyanins, flavonoids and phenolic acids are responsible for at least a portion of the anti-inflammatory and gastroprotective activities of this and many other fruits.

We are presently studying the kinetics of inhibition of these plant extracts and compounds as anti-inflammatory agents and are examining the sites and mechanism of action of iNOS, COX, tumour necrosis factor and other similar compounds (Dominguez *et al.*, 2010).

The use of traditional medicine is widespread and plants still present a large source of novel active biological compounds with different activities, including anti-inflammatory, anticancer, antiviral, antifeedant and antibacterial activities. Together with enzyme inhibitors (inhibitors of tyrosinase, acetylcholinesterase and melanin oxidase), the antioxidants may play a role in health-promoting activity as nutraceuticals (Céspedes, *et al.*, 2006).

1.3 Biopesticides

A widespread effort to find new agrochemicals has focused on natural compounds such as flavonoids, coumarins, terpenoids and phenolics from diverse botanical families from Mexico and the Americas (Alarcon *et al.*, 2011; Céspedes *et al.*, 2006, 2013; Muñoz *et al.*, 2013). In continuation of our general screening programme of the Latin-American flora with biological activities (Céspedes, *et al.*, 2000; Céspedes and Alarcon, 2011), an examination of the extracts and compounds from several selected species such as *Araucaria araucana* (Araucariaceae), *Aristotelia chilensis, Condalia mycrophylla, Talguenea quinquenervis, Discaria spp., Colletia spinossisima* (Rhamnaceae), *Calceolaria integrifolia s.l.* complex (Scrophulariaceae), *Penstemon gentianoides* and *Penstemon campanulatus* (Plantaginaceae, formerly Scrophulariaceae), *Yucca periculosa* (Agavaceae), *Baccharis magellanica, Baccharis salicifolius, Baccharis linearis, Gutierrezia microcephala, Gutierrezia gayana, Roldana barba-johannis, Podanthus ovatifolius, Podanthus mitiqui, Tagetes lucida* (Asteraceae), and two exotic species *Rhus javanica* (Anacardiaceae) and *Pimpinella anisum* (Umbelliferaceae), for biocidal activity has been initiated.

Tyrosinase, also known as polyphenol oxidase (PPO) (Mayer, 2006), is a copper-containing enzyme that is widely distributed in microorganisms, animals and plants. This enzyme catalyses two distinct reactions of melanin synthesis (Robb, 1984): the hydroxylation of a monophenol (monophenolase activity) and the conversion of an *o*-diphenol

to the corresponding *o*-quinone (diphenolase activity). Tyrosinase is responsible for browning in plants and is considered to be deleterious to the colour quality of plant-derived foods and beverages. Tyrosinase also is one of the key enzymes in insect metamorphosis and is involved in sclerotization and moulting regulation processes (Andersen, 1990).

Acetylcholinesterase (AChE), an enzyme contained in nerve tissues, plays a crucial role in the transmission of nerve impulses. Free acetylcholine, the inactive form, is bound to proteins and accumulates at nerve endings in vesicles. As acetylcholine is consumed it is constantly replenished by acetylation of choline. All these processes occur when an impulse is transmitted through a cholinergic synapse. Thus, the process of synaptic transmission is an involved biochemical cycle of acetylcholine exchange. AChE has a key role in this cycle because inhibition of activity leads to the accumulation of free acetylcholine in the synaptic cleft, disrupting nerve impulses. This is followed by convulsive activity of the muscles that can be transformed into paralysis; other features of self-poisoning by surplus acetylcholine then also appear. Some terpenoids are known to inhibit AChE (Ryan and Byrne, 1988; Keane and Ryan, 1999; Miyazawa *et al.*, 2000). Resistance to some insecticides is known to arise by modifications of AChE in insects (Fournier *et al.*, 1994).

In addition to many flavonoids, stilbenoids, phenylpropanoids and phenolics possessing tyrosinase inhibitory activity, many of the same compounds also show strong antioxidant activity in a series of *in vitro* antioxidant assays such as DPPH, ABTS, Trolox, TRAP, ORAC and FRAP. The activity is principally due to the presence of diverse moieties in the chemical structure of the molecules, for instance orcinol or catechol groups, or a hydroxyl group bonded to an aromatic system (gallic acid and gallates in general, resveratrol and other stilbenes, phenylpropanoids, flavonoids, such as quercetin, and other phenolic acids). In these cases, it is possible to correlate antioxidant activity with tyrosinase and AChE inhibition, and IGR activity (Grundy and Still, 1985; Baldwin *et al.*, 2001; Kessler and Baldwin, 2002; Schultz, 2002; Kubo *et al.*, 2003a,b; Torres *et al.*, 2003; Guerrero and Rosell, 2004, 2005).

In addition, many polyphenolic secondary compounds are ubiquitous in angiosperms and have antifeedant effects on phytophagous insects (Feeny, 1976; Rhoades and Cates, 1976; Champagne *et al.*, 1989, 1992; Simmonds, 2003). It has been assumed that phenols bind to proteins, acting as nutritional protein precipitating agents, thus reducing their digestibility (Feeny, 1976; Rhoades, 1979; Martin and Martin, 1982, 1983; Martin *et al.*, 1987; Ortego *et al.*, 1999).

Recent studies have demonstrated that many plant species produce and accumulate a large variety of secondary metabolites that provide defence against insect predators (Berenbaum, 1989; Guella *et al.*, 1996; Marvier, 1996; Berenbaum, 2002). One of the best known efforts has focused on limonoids from the family Meliaceae owing to their potent effects on insect pests and their low toxicity to non-target organisms (Koul and Isman, 1992; Kumar and Parmar, 1996; Singh *et al.*, 1997). Some examples are *Azadirachta indica* (Meliaceae) and *Derris elliptica* (Fabaceae) that produce the well-known insecticide azadirachtin and other types of natural compounds such as rotenone, respectively (Gomes *et al.*, 1981; Kraus, 1993, 1995). The main characteristics that account for the successful use of these secondary metabolites as natural insecticides are mentioned above. These properties make them less harmful to the environment than many synthetic insecticides (Camps, 1988; Berenbaum, 1989; Castillo *et al.*, 1998).

Although members of the family Meliaceae are distributed worldwide, only *Melia, Toona, Cedrela* and *Swietenia* species have been studied in detail (Arnason *et al.*, 1987; Champagne *et al.*, 1992; Arnason *et al.*, 1993; Kraus *et al.*, 1993; Govindachari *et al.*, 1995; Chan and Taylor, 1996; Céspedes *et al.*, 2000). These plants have afforded a number of limonoids such as azadirachtin, gedunin, toosendanin, cedrelanolide, mexicanolide, odoratol, anthothecol, nomilin, bussein and entandrophragmin. Azadirachtin

is the best known example (Champagne *et al.*, 1989; Ramji *et al.*, 1996). This compound and its analogues are potent insect antifeedant and ecdysis inhibitors (Govindachari *et al.*, 1995; Kraus, 1995); however, the structural complexity of azadirachtin precludes its synthesis on a commercial scale (Isman, 2006; Isman and Akthar, 2007), leading us to search for new and simpler secondary metabolites with insecticidal activity. We have focused on members of other families including the Agavaceae and Asteraceae, such as *Yucca, Parthenium Roldana, Tagetes* and *Cedrela* species, especially from tropical and subtropical areas of South and Central America.

Some investigations on the sites and mechanism of action of insecticidal or IGR activity report that different phenolic compounds are enzymatic and metabolic inhibitors (Klocke and Kubo, 1982; Kubo and Klocke, 1986; Kubo *et al.*, 1994, 1995; Kubo and Kinst-Hori, 1999a, 1999b; Kubo *et al.*, 2000; Shimizu *et al.*, 2000; Calderon *et al.*, 2001; Panzuto *et al.*, 2002; Kubo *et al.*, 2003a, 2003b). In addition, many of these compounds are polyphenolic secondary compounds that are ubiquitous in angiosperms and that have antifeedant effects on phytophagous insects (Feeny, 1976; Rhoades and Cates, 1976; Champagne *et al.*, 1989, 1992; Simmonds, 2003).

Our field observations indicate that many botanical species from arid and semiarid lands possess strong resistance to insect attack. The aim of our work is to correlate the phytochemical composition with the inhibitory effect on growth and development of *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae), *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae), *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), *Pieris brassicae* L., *Drosophila melanogaster* M., *Epilachna paenulata* Germar, *Epilachna varivestis* Mulsant, *Cydia pomonella* L., *Aegorhinus superciliosus* (Guerin) (Coleoptera: Curculionidae), *Otiorhynchus sulcatus* (Fabricius) and *Ceratitis capitata* (Wiedemann) (Insecta: Diptera: Tephritidae). Many of these species are very common insect pests in North- and South-American crops. The role of the phytochemical compounds as 'chemical messengers' has proved to be important to our understanding of many ecological problems and has led to the development of 'chemical ecology' (Seigler, 1998).

The effects of phytochemical extracts on aspects such as insecticidal and growth regulatory activity, rate of development, pupation time, adult emergence and deformity have been evaluated and compared with those of gallic acid, gedunin and toosendanin (Chen *et al.*, 1995), and anisic acid and *Cedrela* methanol extract, known growth inhibitors of *S. frugiperda* and tyrosinase, respectively (Céspedes *et al.*, 2000; Calderon *et al.*, 2001; Céspedes *et al.*, 2001a,b; Kubo *et al.*, 2003a,b; Alarcon *et al.*, 2011).

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2 Antioxidants from Vegetal Sources: New Research Avenues

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2.1 Reactive Oxygen Species and Antioxidants: so Far so Good

Free radicals are short-living and highly reactive atoms or molecules with unpaired electrons. In biological systems, the most important radicals are derived from oxygen (reactive oxygen species; ROS) and nitrogen (reactive nitrogen species; RNS). These free radicals are normally generated in cells as products of metabolism and respiratory activity. ROS comprise radical and non-radical oxygen-containing molecules that display high reactivity towards proteins, lipids and nucleic acids. ROS exist in many different inter-convertible forms (e.g. H_2O_2, $O_2^{\bullet -}$, hydroxyl radical, peroxynitrite, etc). Since 1956, when Harman stated that free radicals are involved in cell ageing, a plethora of studies has been carried out with the aim to understand how such compounds cause deleterious effect on biological molecules (Harman, 1956, 1988, 1992). Harman defined the mitochondria as a biological clock and his statements are the basis of the so called 'mitochondrial hypothesis of ageing'. Strictly speaking so did the Argentinian researcher Rebecca Gerschman who, on the basis of the work of Michaelis simultaneously published in *Science* (1954), published the manuscript entitled: 'Oxygen poisoning and X-ray irradiation: a mechanism in common' (Gerschman *et al.* 2005). Through the decades, the contribution of Gerschman and pupils to the field of free radicals has been recognized. Gerschman, in conjunction with Gilbert, developed their theory, which could be summarized in three points: (i) oxygen free radicals are the common mechanism of oxygen and irradiation toxicity; (ii) oxygen toxicity appears equally when oxygen pressure is increased or when there is a decrease in the antioxidant defences; and (iii) oxygen toxicity is a continuous phenomenon contributing to determining lifespan.

Depending on concentration and physicochemical features of the milieu, ROS can be either 'friends' or 'foes'. In order to understand how these species could act as cell signalling molecules (messengers), their physiological functions are currently under study. For many years, the Harman and Gerschman hypothesis has prevailed and the fact that ROS production is a central event in the ageing process is still widely accepted. So, in biological systems, an imbalance in ROS generation might lead to cell death by apoptosis and necrosis (Dypbukt *et al.*, 1994;

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Andersen, 2004; Brookes *et al.*, 2004). The cumulative damage to DNA and proteins has been observed in several pathologies including cancer, atherosclerosis, diabetes and Alzheimer's and Parkinson's diseases (Dhalla *et al.*, 2000; Andersen, 2004; Klaunig and Kamendulis, 2004; Halliwell, 2006a,b). Such accumulative and systemic ROS-induced damage also underlies cell senescence and ageing. Increasing evidence indicates, however, that homeostatic and physiological levels of ROS are indispensable in regulating diverse cellular processes including ion channel/transporter function (Zima and Blatter, 2006), Ca^{2+} spark production (Isaeva *et al.*, 2005; Yan *et al.*, 2008), protein kinase/phosphatase activation, and gene expression (Droge, 2002). The emerging view is that ROS contribute to multiple essential intracellular signalling processes ranging from cell metabolism to ischaemic preconditioning (Droge, 2002; Otani, 2004; Finkel, 2011). Therefore, although the role of ROS as effectors in many processes linked to ageing is recognized, many reasonable doubts have emerged questioning their regulatory functions. Obviously, these doubts also compromise the role of antioxidants in the ageing process. Some authors therefore argue that massive doses of antioxidants do not actually have a significant impact in extending the lifespan of the human population. This is in contrast to the well-known benefits of antioxidant-rich foods consumption, which could improve life quality. For instance, in populations with antioxidant-rich diets a lower relative risk to develop cardiovascular diseases has been reported than for those with a low intake of natural antioxidants contained in fruit and vegetables (Joshipura *et al.*, 1999; Bazzano *et al.*, 2002; Hung *et al.*, 2004). It must be considered, however, that antioxidants act in synergy with other vegetal macronutrients and micronutrients. Interestingly, the search for antioxidant molecules is continuous, independent of the debate related to their relevance. So, their use in pharmaceutical, alimentary (functional foods) and cosmetic industries is a common practice.

Recently, a group of investigators from the Stanford University found that the expression of *elt-3, elt-5* and *elt-6* genes changes during the normal ageing process of *Caenorhabditis elegans* (Budovskaya *et al.*, 2008). These genes were previously identified as responsible for the production of the erythroid-like transcription factors: EL-3, ELT-5 and ELT-6. The regulatory function of these factors is strongly associated with the lifespan of *C. elegans*. Authors found no evidence that age regulation of the *elt-3* transcriptional activity circuit is caused by cellular damage or environmental stresses. Moreover, they observed that *elt-3* expression in adult worms is controlled by increased expression of the repressors *elt-5* and *elt-6*. In summary, the authors propose that a change in the regulation of these genes is caused by age-related drift of an intrinsic developmental programme that becomes imbalanced in old age. These results must be considered with care, however, because worms only live 3 weeks and humans could reach 70 or more years. In line with these results, Bonawitz *et al.* (2007) found that the activity of the target of rapamycin (TOR) negatively regulates lifespan in yeast (Bonawitz *et al.*, 2007). The TOR pathway is a major nutrient-sensing pathway that, when genetically downregulated, increases lifespan in evolutionarily diverse organisms including mammals. In the presence of glucose, the deletion of the *TOR1* gene leads to an increase in mitochondrial activity, promoting chronological lifespan (reviewed by Stanfel *et al.*, 2009). Interestingly, such respiratory activity does not lead to cellular ROS production, because oxidation of the mitochondrial electron transport chain is facilitated. So, intramitochondrial oxygen levels are low, limiting ROS production and accumulation. Stanfel and coworkers (2009) propose that pharmacological inhibition of the TOR pathway should mimic the results observed with *tor1*Δ cells. The central component of this pathway, TOR kinase, is the target of the inhibitory drug rapamycin (Fig. 2.1), a highly specific and well-described drug approved for human use. The investigators found that feeding rapamycin to adult *Drosophila* produces a lifespan extension that mimics those observed in

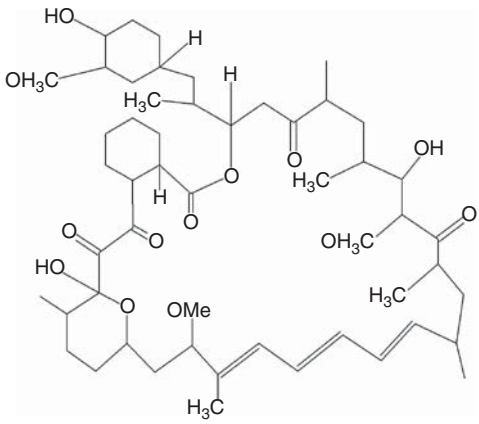

Fig. 2.1. The structure of rapamycin.

some TOR mutants (Bjedov *et al.*, 2010). Moreover, Harrison *et al.* (2009, 2010) found an increase in lifespan when rapamicyn was administrated to genetically heterogeneous mice.

The mutation of a single gene in chromosome 13 causes an extensive ageing phenotype including arteriosclerosis and ataxia, and vascular calcification (Kuro-o, 2000, 2001, 2007, 2009, 2010a,b). This gene was called Klotho and, conversely, its over expression extended lifespan in mice. The Klotho protein functions as a hormone that represses intracellular signals of insulin and insulin-like growth factor (IGF-1), and attenuates ageing. As a hormone, Klotho could circulate and many functions have been described. Among them, this hormone could regulate the production of nitric oxide, the p53/p51 pathway, reduce the angiotensin II-induced ROS production, inhibit Wnt signalling and increase resistance to oxidative stress (Wang and Sun, 2009). Klotho is only expressed in some tissues, but Klotho deficiency impacts nearly all tissues and organs. So, many molecular aspects of Klotho functions remain obscure. For instance, it is possible that some receptors involved in Klotho effects are still waiting to be discovered.

The antioxidant consumption associated with certain foods has proven benefits and many researchers promote its administration through nutraceuticals and functional foods. This supposition must consider that some (perhaps many) biological effects of polyphenols could not necessarily be linked to their antioxidant capacity. In conjunction, these recent studies suggest that ROS are only a part of the picture and it seems that ageing is a highly regulated process, when apparently each individual or species has an 'expiration date'. Therefore, if we want to extend this 'expiration date' by consuming antioxidants we must keep in mind that this only represents a little help.

2.2 Changing the Viewpoint: the Ying and Yang of Antioxidant Molecules

Nowadays it is clear that polyphenols are very promiscuous molecules affecting different biological functions in many ways. Indeed, growing evidence points to the fact that under special conditions these compounds could have pro-oxidant properties. The mechanism underlying this phenomenon has been investigated by several authors (Arakawa *et al.*, 2002, 2004; Aragawa *et al.*, 2003; Aoshima *et al.* 2005). Using green tea catechins as a model, Arakawa and coworkers (2004) demonstrated that at pH 7–8 (or higher) such compounds could generate significant amounts of hydrogen peroxide. The hydrogen peroxide generation might explain the bactericidal effect of certain flavan-3-ols in aqueous media. Also, the production of hydrogen peroxide has been observed in black, green and Oolong teas with concentrations of 1.5×10^{-4}, 2.4×10^{-4} and 0.87×10^{-4} M, respectively. Such H_2O_2 levels are sufficient to exert bactericidal effects against Gram-positive and Gram-negative bacteria (Arakawa *et al.*, 2004). Although the cytoprotective and antioxidant abilities of these compounds are evident in many cell lines, in others they could induce death by apoptosis (Wang *et al.*, 2000a; Lu *et al.*, 2002). This divergence has been observed, for example, in the case of epigallocatechin gallate (EGCG). This compound could generate different oxidative environments protecting normal host cells from ROS damage and, on

the other hand, promoting apoptosis of tumour-derived cell lines (Yamamoto *et al.*, 2003).

Investigating *in vivo* antioxidant properties of natural polyphenols has become a complex challenge for many researchers. The sound case reported by Lotito and Frei (2004) is emblematic. They reported that, after whole-apple consumption, serum antioxidant capacity was significantly increased. They demonstrate, however, that such elevation was actually associated with increased serum levels of uric acid. Moreover, this uric acid derives from the metabolic processing of the fructose contained in apples. In addition, recent findings published in *Hypertension* (Webb *et al.*, 2008) suggest that anti-hypertensive, vasoprotective and anti-platelet effects of many fruits could be associated with their nitrate concentration. In fact, in healthy volunteers, oral intake of beet juice (500 ml) produced a significant lowering of blood pressure, which in all cases was coincident with a plasmatic peak of nitrate. Also, a positive effect of the intake of beet juice on endothelial function was observed. Webb and coworkers (2008) proposed that entero-salival reconversion (promoted by anaerobic bacteria located in the tongue surface) of nitrate-to-nitrite helps to further the production of nitric oxide (NO) in the acidic gastric environment. At the portal level, nitric oxide can be re-oxidized into nitrite and finally converted to NO, particularly in sites where vessel stenosis exists. An increase in the levels of NO could render additional benefit in cases where gastric mucosa is injured by long-term treatment with non-steroidal anti-inflammatory drugs (NSAID) or infected with highly virulent strains of *Helicobacter pylori.* Moreover, it has been observed that in the stomach certain polyphenols promote a rapid NO non-enzymatic formation causing relaxing effects on the vascular smooth muscle (Rocha *et al.*, 2009). An elevation in NO derived from nitrite previously generated by the tongue bacteria (Peri *et al.*, 2005) has been reported for apples (apple pulp is rich in chlorogenic acid). Hence, growing evidence confirms that some of the biological activities of polyphenols do not require high plasmatic concentrations. These data suggest to us that the first (even the most relevant) site of action for these molecules is the gastrointestinal tract (see above).

2.3 Is the Intestinal Tract the First Target for Polyphenolic Antioxidants?

It is well known that antioxidant activity seems to be associated with specific vegetal species used for medical or food purposes. Moreover, such properties are limited to a few families of secondary metabolites. For example, anthocyanidins are common to many berries, whereas glucosinolates are exclusive to cruciferous plants (broccoli). Among these substances, polyphenols are the most consumed. As said above, the data relating to the absorption, metabolism and excretion of polyphenols are poor and very contradictory so many researchers doubt their systemic antioxidant effects after oral intake. So, it has been suggested that the first and maybe the most relevant site of antioxidant action of dietary polyphenols is the gastrointestinal tract (reviewed by Clifford, 2004). Several studies have demonstrated that only certain polyphenols could be intestinally absorbed. Also, it should be considered that only a part of the bioavailable polyphenols circulate in its free forms reaching 5–10% of the initial ingested portion. Hence, polyphenols circulate as conjugated forms such as glucuronides, sulfates and methyl derivates. Nevertheless, the concentration of these conjugated forms still is extremely low.

It is therefore very difficult to carry out real pharmacokinetic studies because normally urine or serum samples must be pretreated with α-glucosidase and/or sulfatase in order to release the aglicones. Such pseudopharmacokinetic studies had led to the conclusion that polyphenol serum concentrations are low, very variable and with transient maxima (T_{max} = 1–2.5 h). Overall, it is unlikely that conjugate forms exceed concentrations of about 10 μM or 1 μM in the case of aglicone forms. Because it is clear that much of the polyphenols are not

absorbed, then it is valid to ask the question: what role is played by this great load of antioxidants in different portions of the digestive tract?

Recently Selma and coworkers (2009) have published a review in which the universe of potential interactions and reactions between polyphenols and intestinal biota is brilliantly illustrated. In this work it was suggested that the systemic effect of polyphenols would be attributable to modulations in the intestinal biota balance and also to the intestinal metabolites generated from these antioxidant molecules. Therefore, the intestinal biota emerged as a key factor behind the observed variability in the results of some studies undertaken in animal and humans (Fig. 2.2).

An example of how the presence of polyphenols in the intestinal tract can bring health benefits is their effect on the pathogen *H. pylori*. The presence of virulent strains of *H. pylori* invariably promotes an immune response characterized by robust oxidative stress as one of the main defence mechanisms against this bacterium. Many of the ROS produced during this process are able to damage gastric and intestinal mucosa. It therefore seems very important to have efficient antioxidant systems (both endogenous and dietary) in the site of infection. However, *H. pylori* have many survival strategies against oxidative stress that give rise to the question: is it good to administrate antioxidants to *H. pylori*-infected individuals? One can deduce that these compounds may have a protective effect not only for the host, but also on *H. pylori* (contributing to their antioxidant defences). Theoretically, by increasing the ability of bacteria to 'deal' with ROS generated by the host immune system, it would also increase the possibility of consolidating infection. Many studies with antioxidant-rich plant extracts have, however, shown *in vitro* and *in vivo* anti-*H. pylori* effects (Mahady *et al.*, 2005; Nostro *et al.*, 2006; Ustun *et al.*, 2006). According to preliminary data, some polyphenolic compounds have recognized bactericidal activity, probably associated with a non-specific mechanism not necessarily related to their antioxidant (cytoprotective) effect on epithelial cells (Puupponen-Pimia *et al.*, 2001). One hypothesis that has been accepted as a paradigm is that polyphenols exert part of their antimicrobial activity by a non-specific interaction with plasma membrane components (Mori *et al.*, 1987; Haraguchi *et al.*, 1998; Funatogawa *et al.*, 2004).

Another attractive approach is how polyphenols can neutralize some virulence factors of *H. pylori* such as urease, VacA and CagA, and modulate the adhesion of this pathogen to the gastric mucosa. *In vivo*, 15–20% of *H. pylori* attach to gastric epithelial cells (Hessey *et al.*, 1990). Because of that, adhesion mechanisms present a very attractive molecular target for limiting *H. pylori* gastric colonization. Indeed, the anti-adhesion properties of

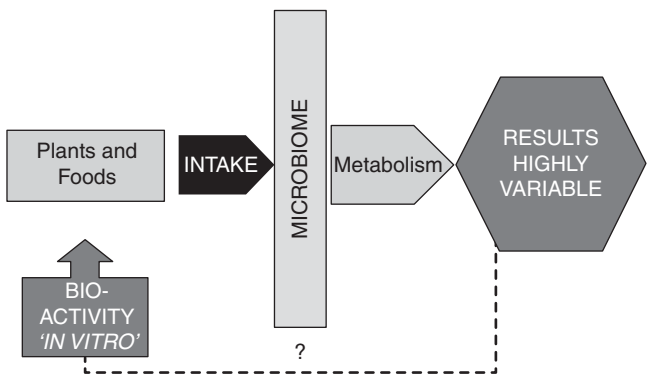

Fig. 2.2. Human microbiota, which can metabolize plant and food constituents, cause highly variable results in clinical trials.

certain polyphenols such as proanthocyanidins have been investigated. Proanthocyanidins, also called condensed tannins, are polymers that have a long history of use in tanning of animal skins, and they are determinants of flavour and astringency in teas, wines and fruits.

Cranberry juice has been studied for its anti-*H. pylori* properties. Preliminarily, Burger and coworkers (2000, 2002) reported that high molecular weight constituents of cranberry juice inhibited the sialic specific bind of *H. pylori* to gastric mucus and mucosal cells *in vitro*. Importantly, this inhibitory effect also was observed for metronidazole- and clarithromycin-resistant strains of *H. pylori* (Chatterjee *et al.*, 2004; Shmuely *et al.*, 2004). The efficacy of cranberry juice against *H. pylori* was tested clinically in a study with 189 infected patients (Zhang *et al.*, 2005) and also in combination with antibiotics and proton pump inhibitors (Shmuely *et al.*, 2007). One half of these individuals (*H. pylori* positive by a ^{13}C-urea breath test) drank 250 ml of cranberry juice daily for 90 days, whereas the others received a placebo drink. After 35 and 90 days, fewer patients tested positive for *H. pylori* in the cranberry juice group.

The intake of cranberry juice in combination with probiotics (*Lactobacillus johnsonii* La1) has been investigated recently by Gotteland and coworkers (2008). In this study, 295 asymptomatic children (6–16 years old) who tested positive for *H. pylori* by the ^{13}C-urea breath test (UBT) received cranberry juice, La1, placebo or a combination of cranberry juice and La1. Eradication rates were significantly different in the four groups suggesting that a regular intake of cranberry juice or La1 could help with the management of *H. pylori* infection in children. Also, some studies reported that cranberry juice associated with other antioxidant-rich sources (*Vitis vinifera*; blueberries and oregano) inhibits the grown of *H. pylori* in a synergistic fashion (Lin *et al.*, 2005; Vattem *et al.*, 2005a). Rohdewald and Beil (2008) demonstrated recently that a pine bark proanthocyanidin-rich extract (Pycnogenol®) concentration-dependently inhibited the *H. pylori* adhesion to AGS cells.

On the basis of the above findings, our group recently evaluated an apple peel polyphenol-rich extract (APPE, 24% procyanidins), against *H. pylori* adherence and vacuolation in HeLa cells (Pastene *et al.*, 2010). We found that APPE exerted dual anti-*H. pylori* effects, inhibiting the process of adherence of the bacteria to gastric mucosa and also the activity of VacA protein. The anti-adherence effect was limited, however, because the mean degree of polymerization (*DPm*) of APPE is ~3. This *DPm* is lower than that reported for other sources such as pine bark (8–10) or persimmons (19–47) (Jerez *et al.*, 2007; Li *et al.*, 2011). So, the APPE anti-adherence effect was observed at an IC_{50} value of 5.3 mg of gallic acid equivalents per ml (GAE/ml). This result suggested to us that the source and methodology of procyanidin extraction are very important. Using solid-state bioprocessing (with *Rhizopus oligosporus and Lentinus edodes* fungi), a group of researchers were able to increase the extraction yield of polyphenols from cranberry pomace, obtaining a product with higher anti-*H. pylori* activity (Vattem *et al.*, 2005b). In a similar approach, from bio-processed pineapple wastes (*Ananas cosmosus*), other researchers found extracts (enriched in quercetin and biphenyl structures) with a potent anti-*H. pylori* effect (Correia *et al.* 2004). In the latter study, however, the antioxidant capacity of the extracts did not correlate with the anti-*H. pylori* activity.

In a recent study, the antimicrobial activity of 12 types of Nordic berries was evaluated on various human pathogens. In this work, *H. pylori* and *Bacillus cereus* were the most sensitive pathogens assayed. Interestingly, although it was observed that the levels of polyphenols decreased during cold storage, the antimicrobial activity was not significantly modified (Nohynek *et al.*, 2006). Considering the elevated polyphenolic contents found in tea, its anti-*H. pylori* effects also have been investigated. *In vitro* studies using 5% infusions of green tea (*Lung Chen*, cv) showed inhibition of *H. pylori* multiplication, suggesting that such activity would reside in the presence of the flavan-3-ol known as epigallocatechin

gallate (EGCG) (Yee and Koo, 2000). The same team of investigators subsequently performed a prospective study between two groups of individuals: one *H. pylori*-infected (n = 42) and one uninfected (n = 30). The main outcome was the effect of tea consumption on the detection of *H. pylori* in gastric biopsies. It was concluded that there would be a significant inverse relationship between tea consumption and the rate of infection with *H. pylori* (Yee *et al.*, 2002).

One study focused on the evaluation of potential antimicrobial activity of apple peel polyphenols (Alberto *et al.*, 2006). Although this work shows that this extract exhibits certain anti-*Escherichia coli*, anti-*Pseudomonas aeruginosa* and anti-*Staphylococcus aureus* activity (among others), it did not include *H. pylori* in the evaluation. Apple peel polyphenols display interesting mechanisms that prevent colonization by *H. pylori* in mice. For example, their procyanidins can inhibit efficiently the enzyme urease and neutralize VacA, whereas its flavonoids (quercetin glycosides) have both anti-*H. pylori* and anti-ROS effects contributing to avoiding damage to gastric mucosa (Pastene *et al.*, 2009a,b, 2010). Previously it was reported that flavonoids can inhibit *H. pylori*-induced vacuolation in HeLa cells and additionally display a moderate inhibitory activity against urease (Shin *et al.*, 2005). In the case of procyanidin-rich extracts from *Vitis vinifera*, Lee *et al.* (2006) found that these compounds are particularly active in inhibiting urease at low concentrations (0.1 mg/ml).

Apples also have other phenolics with anti-*H. pylori* activity such as phloridzin (a chalcone). This compound inhibits the pore-forming ability of the VacA toxin. However, the anti-VacA activity (IC_{50} = 273 μM) of phloridzin is extremely low compared with activity reported for tannic acid (IC_{50} = 2.7 μM). In fact, hydrolysable tannins are more powerful than other polyphenols. In another study, it was established that tannic acid associated with n-propyl gallate was very effective in inhibiting gastritis promoted by *H. pylori* infection or direct administration of VacA to mice (Ruggiero *et al.*, 2006). To investigate the proposed mechanism, the structure–activity relationships between polyphenols and their anti-VacA activity (expressed as vacuolizing activity and urea transmembrane flux) were assessed in HeLa cells (Tombola *et al.*, 2003; Ruggiero *et al.*, 2006). Resveratrol, morin, tannic acid and piceatanol seem to share structural features that suggest the existence of specific molecule–molecule interactions between certain polyphenols and VacA.

Other polyphenols with high antioxidant activity such as ellagic acid and myricetin were partially or totally inactive against VacA, respectively. These compounds are present in varying amounts in many foods and can be found in high concentrations in wine, beer, chocolate and green tea. Shin *et al.* (2005) demonstrated that antioxidants with diverse structures such as ascorbic acid, glutathione, epicatechin and Trolox® (a water-soluble analogue of α-tocopherol) have in common that they are poor VacA inhibitors, suggesting that antioxidant and anti-*H. pylori* activities are not necessarily associated. Among these antioxidants, the *H. pylori* inhibitory activities of epicatechin and vitamin C were evaluated. In this study, only vitamin C was active *in vitro* and *in vivo* by a mechanism not yet clarified, but in any case this would not be associated with the effect of pH (Zhang *et al.*, 1997; Mabe *et al.*, 1999; Wang *et al.*, 2000b). The literature reports another relevant fact that some flavonoids inhibit the VacA-induced activation of procaspase-3 to caspase-3 without changes in the expression of proteins Bax and Bcl-2 (anti-apoptotic proteins). Thus, it has been suggested that flavonoids such as quercetin can protect gastric cells from apoptosis by inhibiting the action of vacuolating *H. pylori* toxin VacA. Additionally, it was found that certain high molecular weight polyphenols (oligomeric procyanidins extracted from the hop bract), some of which are structurally closely related to apple peel polyphenols, are capable of forming complexes with VacA *in vitro* (Yahiro *et al.*, 2005; Friedman, 2007). Yahiro *et al.* (2005) observed that the interaction between oligomeric proanthocyanidins (with a mean degree of polymerization equivalent to 22 catechin units) and

VacA toxin represents a potential mechanism of neutralization for the different virulence factors of this bacterium. These compounds were effective in blocking the binding of VacA to its receptors RPTPα and RPTPβ, inhibiting the non-specific binding of VacA to cell membranes, decreasing the cell vacuolation *in vitro* and significantly diminishing VacA-induced gastritis in mice. Likewise, it was demonstrated that the administration of red wine extract and/or green tea promoted a clear gastroprotective effect in mice infected with *H. pylori* or treated with purified VacA. These data suggest that VacA would be a potential molecular target for certain wine and green tea polyphenols (Ruggiero *et al.*, 2007). Interestingly, it was found that quince pulp extracts compared with apple pulp extracts have a higher antiulcerogenic effect on ethanol and HCl-induced gastric injury (Hamauzu *et al.*, 2006). The authors suggest that the difference in the effectiveness of such antiulcerogenic extracts lie in the significantly higher concentration of procyanidins, with a high degree of polymerization present in quince pulp (*DPm* = 29) versus apple pulp (*DPm* = 3). Unlike apple pulp, however, peel is particularly rich in procyanidins with high mean degree of polymerization (*DPm* = 15–190) (Shibusawa *et al.*, 2001).

Saito *et al.* (1998) previously reported that seed extracts of *Vitis vinifera* and its procyanidins (with a high molecular weight) have an anti-ulcerogenic effect; the authors postulated that part of this effect is due to the strong binding of these compounds to proteins present in the gastric mucosa, allowing the formation of a protective local barrier with potential antioxidant and anti-inflammatory activities. Indeed, it was recently established that polyphenol-enriched extracts of apple pomace promote antioxidant and cytoprotective effects in primary cultures of gastric mucosal cells (MKN-28) subjected to a xanthine–xanthine oxidase system as a superoxide radical generator (Graziani *et al.*, 2005). The protective effect of these extracts has been observed in *in vitro* (Caco-2, AGS cells) and *in vivo* models of injury induced by co-administration of indomethacin (Graziani *et al.*, 2005; Carrasco-Pozo *et al.*, 2010, 2011a,b). Interestingly, oxidative damage and gastric mucosal lesions induced by indomethacin are exacerbated by infection with *H. pylori* (Arend *et al.*, 2005). In another study, it was established that apple peel extract inhibited, *in vitro* and *in vivo*, the activation of the transcription factor AP-1 and neoplastic transformation (Ding *et al.*, 2000). The activation of the AP-1 complex is one of the key events in tumour promotion mediated by the cytotoxin CagA of *H. pylori*. It has been suggested that apple polyphenols could inhibit the activation of the AP-1 signalling pathway by interfering with the MAP kinases ERK and JNK. In electron spin resonance (ESR) experiments, Ding and colleagues (2000) confirmed the stabilizing effect of apple peel extract on $OH^{\bullet}$ and $O_2^{\bullet -}$ radicals. The latter would be particularly relevant given that ERKs, JNKs and p38 are molecules activated in response to oxidative stimuli. Finally, the same authors showed these extracts also inhibit tumour induction in mice treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). The diverse molecular targets for polyphenols during the *H. pylori* infection process are summarized in Fig. 2.3. Hence, polyphenols could display their effects against *H. pylori* not only affecting its viability but also its adherence to gastric mucosa (1). Certain polyphenols may have a mucosa cytoprotective effect (2). The effect of polyphenols against some virulence factors like VacA (3) or urease (4) could be explained by the formation of inactive complexes. Finally, antioxidant and anti-inflammatory effects associated with polyphenol intake could be explained by a inhibition of ROS and interleukin (IL-8) production in those neutrophils activated by *H. pylori* (5).

2.4 Polyphenols: What we Learn Measuring the Antioxidant Capacity

The view on the importance of polyphenols in human health has been extensively revised (Kris-Etherton *et al.*, 2004; Manach *et al.*, 2005; Williamson and Manach, 2005;

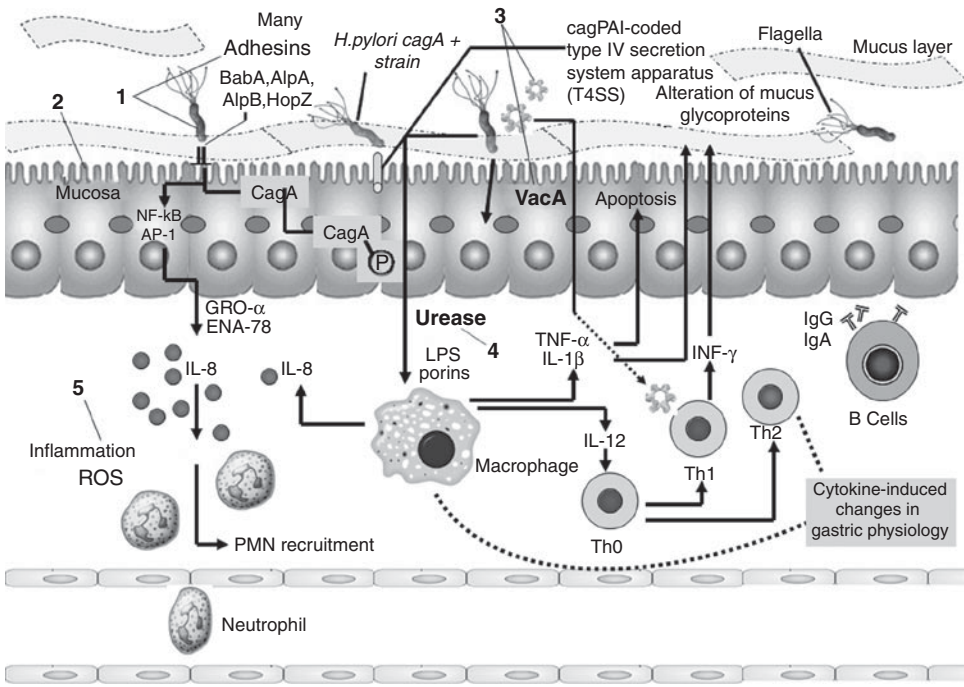

Fig. 2.3. Potential sites of action of polyphenols as cytoprotective agents and anti-*H. pylori*. Numbers in bold represent the effects detailed in the text.

Ramassamy, 2006; Perron and Brumaghim, 2009; Chong *et al.*, 2010; Ostertag *et al.*, 2010; Vauzour *et al.*, 2010; Weseler *et al.*, 2011). A crucial aspect of research on the antioxidant capacity of natural products is, however, the correct choice of the measurement tools. The state of the art approach indicates that it is becoming necessary to have a battery of tests that allow us to obtain complementary information. In this regard, the use of coloured stable radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), N,N-dimethyl-p-phenylenediamine (DMPD), or reagents such as ferric reducing antioxidant power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC) are recommended as a preliminary ranking criterion for different vegetal sources, extracts or fractions thereof, according to their antioxidant power. Electron transfer and/or hydrogen reactions occur in these assays. Adequate knowledge of the chemistry of such systems is therefore a prerequisite for correctly interpreting the results, particularly when these tests are applied to highly complex samples such as biological fluids. In the following sections we will review some of the methods used for the determination of antioxidant capacity with emphasis on those considered most useful and innovative.

2.5 Antioxidant Assays using Cell-free Media

As shown in many studies, data of antioxidant capacity for plant and foods are often crossed with the total polyphenol content, determined as gallic acid equivalents (GAE). It is, however, increasingly frequent recourse to separation techniques coupled to online detection systems, such as diode array (DAD) or mass spectrometry (LC-MS). Such techniques help to better dissect the antioxidant activity and assign it to a specific

group of compounds. One of the radicals most used as a probe in cell-free systems is DPPH because it is very easy to use, fast and inexpensive and can be used in any laboratory with minimal equipment. Because the chemistry behind the reactions of DPPH with different antioxidants still requires some understanding, the results should be interpreted with some care. Additionally, it should be considered what type of solvent is applied in the assay, and the effect of pH, temperature, light, reading time and the concentration of the radical. The mathematical treatment of results deserves special mention. Traditionally, the percentage of inhibition is calculated at an arbitrary end time. Recent studies indicate that measuring time should not be less than 20 min; it is recommended to use the area under the curve of DPPH bleaching for the calculation of this parameter (Cheng *et al.*, 2006). The use of Trolox as a calibration substance is desirable to calculate Trolox equivalent antioxidant capacity (TEAC) values, which may be useful for comparison purposes. Other antioxidants such as vitamin C, gallic acid, catechin and quercetin can also be used for calibration purposes. Considering the DPPH bleaching rate in the first few seconds (fast kinetic) or after 5 min (slow kinetic), as in the ABTS assay (see below), it is possible to calculate two TEAC indexes (Campos and Lissi, 1995, 1997). Detailed analysis of both portions of these curves can provide interesting information about the quality and reactivity of the tested antioxidants. In general, the method can be used as a preliminary test in the detection of antioxidant extracts and for monitoring the chromatographic fractions. DPPH can even be used as post-column reagent for high-performance liquid chromatography (HPLC) or to stain thin-layer chromatography (TLC) plates. The case of ABTS radical is analogous to that of DPPH; it provides similar information but must be generated in additional steps. Many ways have been proposed to do this, for example using 2,2´ azo-bis (2-amidinopropane) hydrochloride (AAPH) previously thermo-activated (Henriquez *et al.*, 2002), potassium persulfate (Re *et al.*, 1999) and recently *in situ* by mixing peroxidase/H_2O_2/ABTS (Munoz-Munoz *et al.*, 2010). In the latter work it is worth noting the introduction of a new parameter called the apparent kinetics antioxidant efficiency (KAE_{app}), which would be useful for comparing different antioxidants. Furthermore, the authors introduce the concepts of a primary and secondary antioxidant on the basis of similar observations with DPPH.

2.5.1 ORAC

Although there is no methodology that can be considered a 'gold standard', many studies find that certain tests such as oxygen radical absorbance capacity (ORAC) have comparative advantages over the rest of the assays (Ou *et al.*, 2001). The ORAC index combines in a single parameter information on the kinetics of oxidation using the area under the curve of fluorescence or absorbance decay of a probe such as fluorescein (ORAC-FL) or pyrogallol red (ORAC-PGR), which is challenged with peroxyl radicals (AAPH). In the ORAC-FL assay, the times of induction are strongly influenced by the number of phenolic groups present in the sample, whereas in the ORAC-PGR assay such times are not practically observed and the decay of the absorbance is influenced mainly by the reactivity of the phenols of the sample. Recently, it has been suggested that both ORAC indexes (FL and PGR) are complementary and their ratio is a better indicator of the average quality of the antioxidants contained in a sample (Poblete *et al.*, 2009). The same research group has proposed ORAC-PRG as a quick way to determine the specific content of vitamin C in extracts and biological fluids, because this substance is one of the few that produces induction times in a concentration-dependent manner (Torres *et al.*, 2008; Atala *et al.*, 2009). In the search for the 'methodological Holy Grail' and to expand its range of applications, several refinements have been introduced to this assay. For example, the use of methylated cyclodextrin allows obtaining the lipophilic ORAC index in different plant samples or biological fluids

(Huang *et al.*, 2002). Additionally, the assay has proven its versatility because some changes have been introduced for measuring the effect of antioxidants on reactive species of oxygen and nitrogen, giving rise to variations for the hydroxyl radical (HORAC), peroxynitrite (NORAC) and superoxide anion (SORAC) (Ou *et al.*, 2002; http://www.brunswicklabs.com/). The use of ORAC has been employed by the United States Department of Agriculture (USDA) as a criterion of antioxidant power. The USDA (http://www.ars.usda.gov/nutrientdata/ORAC) has published a series of tables containing not only the food composition of many medicinal plants used in North America but also information about their antioxidant activity. Moreover, in the Laboratory of Antioxidants at the Institute of Nutrition and Food Technology (INTA), University of Chile, a website was recently created where ORAC values and polyphenolic contents of Chilean fruits are presented in a complete database (www.portalantioxidantes.com). This project, led by Dr Hernan Speisky and funded by INNOVA-Chile, makes Chile the only country in Latin America and second in the world to carry out this type of initiative.

2.5.2 FRAP

The ferric reducing antioxidant power (FRAP) assay is one of the methods historically used to evaluate the antioxidant capacity of samples from different origins. In fact, the method measures the ability of a sample to reduce Fe^{3+} to Fe^{2+}. Therefore, the ability to donate electrons from a molecule is reflected by the concentration of ferrous ions in the medium. These ions can be quantified owing to their ability to form an intense blue-coloured complex with 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ). Despite its simplicity and low cost, this method has some important limitations such as a pH under the physiological range (pH = 3.6), and the arbitrariness of reading time. Many of these disadvantages are overcome in the method CUPRAC.

2.5.3 CUPRAC

In recent years an assay called cupric ion reducing antioxidant capacity (CUPRAC) has been developed and successfully used for the determination of antioxidant capacity of samples with polyphenols, ascorbic acid and thiols (Apak *et al.*, 2004, 2007; Cekic *et al.*, 2009). In this assay, antioxidants reduce Cu^{2+} to Cu^{+}. The complex formed between neocuproine and Cu^{+} can be detected spectrophotometrically, and its concentration is proportional to the ability of antioxidants to transfer electrons to copper. Among the main advantages of CUPRAC assay it is worthy to mention that it can be performed in a medium with physiological pH (7.4) and that measurements are registered at an end point (30 min). This method has significant advantages over FRAP because the redox chemistry of copper (II), as opposed to that of the chemically inert high-spin ferric ion with half-filled d-orbitals in its electronic configuration, involves faster kinetics. For this reason, some phenolic antioxidants continue promoting the formation of the TPTZ–Fe^{3+} complex even hours after the study has been completed (typically FRAP readings are performed at 5–10 min). Also it has been established that, owing to lower redox potential of the CUPRAC reagent, reducing sugars (fructose) and citric acid are not oxidized with the CUPRAC reagent. This assay also is applicable to thiol-type antioxidants, which cannot be measured by the FRAP assay. The method has also been adapted for the analysis of hydrophilic and lipophilic antioxidants (Çelik *et al.*, 2007) and in the determination of hydroxyl radical scavenging capacity (Bektasoglu *et al.*, 2008).

2.6 Cell-based Assays

2.6.1 ERYCA

Red blood cells can be used as a simple and inexpensive biological system to determine antioxidant capacity of extracts and pure substances. In the original assay, erythrocytes

are challenged with oxidizing agents such as HOCl, TBHP, H_2O_2 or AAPH, which promote membrane damage leading to haemolysis. Thus, determining the percentage of haemolysis in the presence of increasing concentrations of antioxidants is a good indicator of the antioxidant potential of the sample. Recently, Gonzalez and coworkers developed a new method called erythrocyte cellular antioxidant activity (ERYCA). They proposed a modification of the assay, making turbidity readings at 660 nm, instead of haemolysis (Gonzalez *et al.*, 2010). This allows the conversion of an end-point assay to a kinetic one, where decreases in absorbance (light scattering) versus time plots generated decay in which the area under the curve is calculated in the same manner as in the ORAC assay (Fig. 2.4 a, b). Although this method is not widely used, hypothetically it could be adapted to include other ROS-generating systems. The method is also visually more interesting than the ORAC assay, particularly for the *in vivo* evaluation of antioxidant capacity, lacking only validity in its usefulness, reproducibility and repeatability with more studies.

2.6.2 Study of ROS production in neutrophils, monocytes and other cell lines

Neutrophils are phagocytic cells able to assemble a highly efficient ROS-producing machine to deal with different stimuli. Neutrophils are easily purified from blood collected from laboratory animals or human volunteers. Typically, using double gradient centrifugation with Histopaque 1119 and 1077 it is possible to obtain both neutrophils and mononuclear cells with high purity and viability. Mononuclear cells could be cultured and used to study the production of certain radicals such as NO. Neutrophils have a lifetime of ~4 h and can be stimulated with various agents such as PMA, fMLP, opsonized zymosan, ionophores (ionomycin) and bacteria, among others (Pastene *et al.*, 2009a). Initially, ROS production is governed by the activity of NADPH oxidase. This complex, under normal conditions, is assembled inside the phagosome, where it directs the production of $O_2^{\bullet -}$. This species can subsequently lead to H_2O_2, which in turn is the substrate of another enzyme,

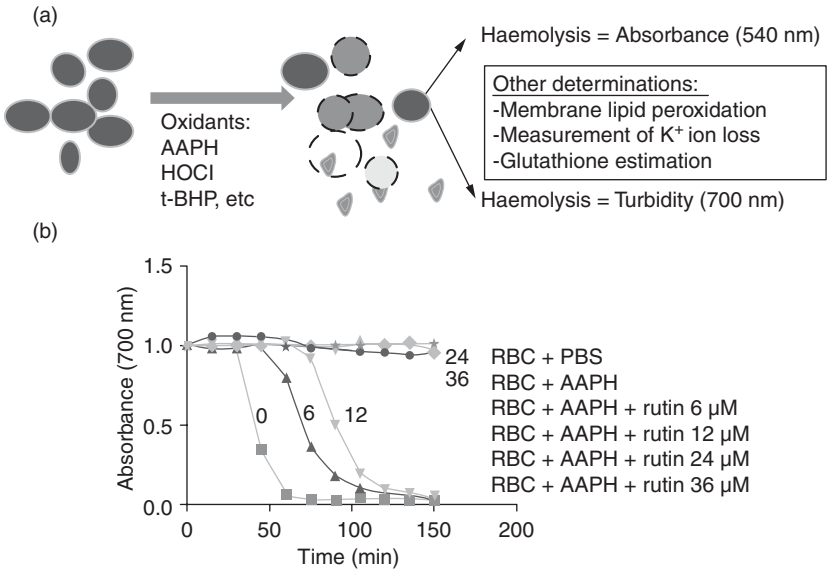

Fig. 2.4. (a) Illustration of the ERYCA assay. AAPH, 2,2´ azobis (2-amidinopropane) hydrochloride; t-BHP, tert-butyl hydroperoxide. (b) Antioxidant effect of rutin (0–36 µM) on AAPH-induced peroxidation on human erythrocytes (RBC) (Aguayo, 2011).

myeloperoxidase (MPO). The latter generates the species HOCl, whose bactericide and oxidant activity is important for the destruction of bacterial agents. Therefore, the neutrophil is a complex but reliable system for the study of antioxidants in biological environments, particularly where it is developing an inflammatory process. For a proper interpretation of the results, we must keep in mind the use of inhibitors and amplifiers of the response. For example, the systems luminol / superoxide (SOD) / catalase (CAT) and isoluminol / horseradish peroxidase (HRP) allow us to obtain information from the production of ROS both within and outside the neutrophil. In the first case, luminol acts as a permeable chemiluminescent probe that is distributed evenly inside and outside the neutrophil. If using the mixture of SOD / CAT, the extracellular production of ROS may be suppressed, leaving only the intracellular production. The latter can be amplified in the presence of luminol. On the other hand, isoluminol is impermeable and therefore can be used to detect extracellular production of ROS. Because such production is strongly linked with MPO released by neutrophils, often it must be reinforced with HRP, because in the first steps of the respiratory burst the amount of MPO in the extracellular environment is minimal. As mentioned below, it must be remembered that the probes used are not always selective enough. Thus, both luminol and isoluminol preferably detected HOCl, showing less sensitivity for the case of superoxide anion and hydrogen peroxide. When looking for a detailed study of the effect on NADPH oxidase activity, and therefore on superoxide anion production, the alternative use of lucigenin is more recommendable. As discussed in the next section, new probes have been developed for the study of specific cellular levels of certain radicals of biological relevance. The use of fluorescent probes derived from fluorescein is a fine approach to detect visually where ROS production occurs. Mononuclear cells are obtained together with neutrophils after the double gradient separation. Usually, these cells require a special culture medium such as RPMI 1640. The production of ROS by monocytes has a certain delay compared with neutrophils when different activators are used. Although monocytes have intracellular granules with peroxidases, they possess less MPO than neutrophils and therefore produce less HOCl. Monocytes could be stimulated to promote differentiation to macrophages, in which it is possible to study the production of $NO^{\bullet}$, generated by the enzyme iNOS. Traditionally nitric oxide can be measured indirectly by the Griess reaction for the oxidation product (NO_2^-), and ultimately by specific electrodes (Gobert *et al.*, 2001; Rocha *et al.*, 2009).

Overall, the production of ROS and RNS can be measured in various cellular contexts and the choice depends on the research problem and the resources of each laboratory. Because of their importance in cardiovascular pathology, human umbilical vein endothelial cells (HUVECs) are an interesting model, but their preparation requires more expertise, which increases the cost of any analysis. For those who study the antioxidant capacity in cell lines it is highly recommended to assess the permeability of the tested molecules in such a context. Many of the effects (including the production of ROS) of polyphenols do not require penetration into a cell.

2.7 Every ROS has its Thorn: New Probes under Investigation

2.7.1 Common probes used to detect ROS in cells

So far, there are few 'gold standard' probes universally employed and specific enough to measure certain free radicals. The reader who wishes to delve into this area can see some good reviews published recently that cover the advantages and limitations of various reagents for the detection of ROS (Freitas *et al.*, 2009; Niki 2010a,b; Rhee *et al.*, 2010). Thus, this section only summarizes some of the most widely used reagents for cellular and non-cellular systems.

Cytochrome c

The reduction of cytochrome c has been widely used to estimate specifically ROS release in neutrophils stimulated with different agents. The reaction is inhibited almost 100% by SOD addition. The product of the reaction: cytochrome c [Fe (III)] + $O_2^{\bullet-}$ → O_2 + cytochrome c [Fe (II)] can be analysed at 550 nm. Although this assay is even less sensitive than lucigenin for determining the extracellular production of $O_2^{\bullet-}$, it basically provides the same information.

Tetrazolium salts: NBT; MTT, XTT, WST-1

All these compounds have in common their ability to be reduced by reactive species to form a highly coloured formazan that can be analysed using spectrophotometry and visualized in cells. These salts are particularly sensitive to $O_2^{\bullet-}$ generating the radical tetrazoinil, which dismute to form the water-insoluble blue formazan. Formazan must be solubilized for its quantitation and therefore attempts have been carried out using MTT or the WST-1 for the same purpose because they produce derivatives with increased water solubility. WST-1 compounds have low cost, less probability of dismutation of $O_2^{\bullet-}$ to H_2O_2 and their reduction is almost 100% inhibited by SOD, suggesting low cell permeability.

Lucigenin

As indicated earlier, lucigenin can be used for its higher selectivity and sensitivity to $O_2^{\bullet-}$ production compared with tetrazolium salts. Superoxide is able to reduce lucigenin to form dioxetane. The latter is unstable and breaks spontaneously to form N-methylacridone in an excited state, which leads to light emission. Because lucigenin is highly selective to $O_2^{\bullet-}$, its detection does not depend on the presence of MPO as it does for luminol. Although lucigenin can promote $O_2^{\bullet-}$ generation by redox recycling, the amount produced is minimal. Lucigenin is not a permeable molecule and therefore when using SOD, the emission of light is practically inhibited by 100%.

Dihydroethidium (hydroethidine)

This probe is cell permeable and can be oxidized by superoxide to form the ethidium cation, which has a strong fluorescence. Because hydroethidine (HE) could be oxidized by other ROS, there is still some debate as to whether the ethidium cation is really a specific product for the presence of $O_2^{\bullet-}$. An HPLC analysis of the HE oxidation products promoted by superoxide revealed a peak corresponding to a new substance, which was assigned to 2-hidroxietidium. The latter has different fluorescence properties to the ethidium cation and its specific recognition should be performed by HPLC-FLD (Zhao *et al.*, 2003, 2005; Fernandes *et al.*, 2007). This compound has a minor tendency to produce superoxide by redox recycling and is used for detection of intracellular ROS. Disadvantages are that it has high photolability and that in cells undergoing apoptosis by the intrinsic pathway the release of cytochrome c may lead to artefactual oxidation of hydroethidine (Zielonka *et al.*, 2008).

Dihydrorhodamine 123 and 2,7-dichlorodihydrofluorescein

Dihydrorhodamine (DHR) is a lipophilic probe sensitive to hydrogen peroxide. Once inside the cell DHR undergoes oxidation and one of its amino groups tautomerizes to the imino form (Rho123), preventing it from leaving the cell (Henderson and Chappell, 1993). 2,7-Dichlorodihydrofluorescein (DCFH) is a derivative of fluorescein that can be oxidized by ROS generating intense fluorescent dichlorofluorescein (DCF). The diacetate derivative of DCFH is apolar and non-fluorescent and thus can enter cells, where it can serve as substrates for esterases to release DCFH. This latter is polar and is therefore trapped inside the cell. The probes respond to both ROS and RNS (Crow, 1997). The main disadvantage of both DHR and DCFH is high sensitivity to photo-oxidation and a certain tendency to leak away from cells in long-term assays. Although both probes could be used, some authors preferred DHR instead of DCFH for H_2O_2

detection in living cells (Qin *et al.*, 2008; Sakurada *et al.*, 1992).

Amplex Red

N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) is a highly specific probe for the detection of hydrogen peroxide produced in neutrophils. When this probe is oxidized by hydrogen peroxide it generates a stable fluorescent product resofurin, which can be analysed using excitation at 520–550 nm and an emission at 585–595 nm. Although this compound is not interfered with by the autofluorescence of biological samples, some vegetal constituents could interfere with Amplex Red oxidation (Serrano *et al.*, 2009; Mishin *et al.*, 2010).

Tetramethylbenzidine

Tetramethylbenzidine (TMB) is a probe used to detect HOCl, generating a blue product when it is oxidized. A disadvantage of this probe is that it can be a substrate of MPO, limiting its applications (Freitas, 2009).

Diaminofluoresceins

These compounds (DAF-2 and DAF-FM) have been used successfully for recognizing the extracellular production of NO. In an analogous way to DCHF-DA, their acetylated derivatives are used for the recognition of intracellular production of NO. These probes are highly sensitive and allow monitoring of NO production in real time.

Ethanol/a-(4-pyridyl-1oxide)-N-tert-butylnitrone (4POBN)

This probe is one of the few existing molecules able to detect specifically the production of the hydroxyl radical.

MitoSOX Red

This is a fluorescent probe specifically oxidized by mitochondrial superoxide. It can be used to investigate the production of ROS in living cells. It should be noted that their oxidation can be prevented by adding SOD-mimetic agents such as Tiron and FeTCPP. Although much hope was placed on this probe, its specific use to detect intracellular superoxide production is controversial. So, Zielonka and Kalyanaraman critically reviewed the reliability of this probe, concluding that it must be used with care and HPLC profiles should be traced in order to observe all HE oxidation products (Zielonka and Kalyanaraman, 2010).

2.7.2 New probes for the detection of ROS

Recently, substantial progress has been made in the synthesis of new and more specific probes to detect ROS.

New probes for the hydroxyl radical (OH)

Traditionally, the hydroxyl radical is detected by reaction with salicylate or phenylalanine (Althaus *et al.*, 1993; Halliwell and Kaur, 1997; Luo and Lehotay, 1997). However, both strategies do not allow a realistic estimate of the production of hydroxyl radical and some new probes have been introduced.

TEREPHTHALATE. The product of terephthalate hydroxylation (2-hydroxy-terephthalate) has a higher fluorescence intensity than 2-hydroxy-benzoate (Saran and Summer, 1999). Because of that, this probe has gained popularity for detecting OH radicals produced from diverse sources, including living systems, in the micromolar range (Freinbichler *et al.*, 2008a,b; Page *et al.*, 2010). Detection could be done using fluorescence readers or, even better, trough HPLC-FLD (Li *et al.*, 2004).

DPPEC. Similarly, the fluorescent probe 1,2-dipalmitoylglycerophosphorylethanolamine (DPPEC) has been developed for the detection of the hydroxyl radical in lipid membranes (Soh *et al.*, 2008). The probe has high selectivity for this radical.

TEMPO. Recently, Maki and colleagues developed a new probe from the union of

perylene-3,4,9,10-tetracarboxyl bisimide and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Maki *et al.*, 2009). This compound emits fluorescence in the visible region and is highly selective for the hydroxyl radical.

RHODAMINE NITROXIDE PROBES. Most recently, Yapici and coworkers developed a series of rhodhamine nitroxide probes (I, II and III) specific for OH that can be used for fluorescence and ESR detection (Yapici *et al.*, 2012). These molecules have been successfully assayed for OH detection in cell-free systems (Fenton reagent), ARPE-19 cell stimulated with PMA and tumour lines such as HeLa, HepG2 and SW-620.

A new probe for superoxide

Circularly permuted yellow fluorescent protein (cpYFP), previously used as the core structure for the Ca^{2+} indicator pericam (Nagai *et al.*, 2001), is a novel biosensor for $O_2^{\bullet -}$, the primal ROS generated by the electron transfer chain. The fluorescence emission (515 nm) of purified cpYFP when excited at 488 nm is five times brighter under strong oxidizing conditions. Extensive *in vitro* experiments revealed the superoxide selectivity of cpYFP over other physiologically relevant oxidants and metabolites. The $O_2^{\bullet -}$ associated increase in cpYFP fluorescence is completely reversed by the subsequent addition of Cu/Zn-superoxide dismutase (SOD, 600 U/ml) or prevented by prior addition of SOD. By contrast, cpYFP emission is unchanged by H_2O_2 and peroxynitrite, and is decreased by $HO^{\bullet}$ and NO (Wang *et al.*, 2008).

New probes for hydrogen peroxide

PEROXIFLUOR-1. In order to improve the specific detection of H_2O_2, Chang and coworkers developed the peroxifluor-1 probe (Chang *et al.*, 2004). Indeed, their response to H_2O_2 is 500 times greater than for other ROS. A family of boronate probes has been synthesized, such as peroxyresofurin-1 (PR-1), greenfluorescent PF-1 and the blue-fluorescent peroxyxanthone-1 (PX1). Interestingly, all these probes are permeable and can detect micromolar concentrations of H_2O_2 *in vivo*. Boronate-derived probe oxidation can be used for studying localization, trafficking and *in vivo* production of H_2O_2 in various living systems (Lippert *et al.*, 2011).

ORGANELLE-SPECIFIC DETECTION OF H_2O_2 USING SNAP-TAG PROTEIN LABELLING. A refining of boronate chemistry has been recently developed. Using SNAP-tag technology, site-specific protein labelling can be done in practically any cell compartment (nucleus, mitochondria, plasma membrane and endoplasmic reticulum). Hence, localized H_2O_2 production has been detected with one of these fusion products named SNAP-peroxy-Green (SNAP-PG). The specificity of this probe was evaluated using scanning confocal microscopy (Dickinson *et al.*, 2010; Srikun *et al.*, 2010).

HYPER. OxyR is a sensor and transcriptional regulator that can detect H_2O_2 through domains that may sense this ROS. The sensor domain is called OxyR-RD, and by fusion with cpYFP gives rise to the Hyper. This probe has two excitation peaks at 420 and 500 nm, with emission at 516 nm. When exposed to H_2O_2, the 420 nm peak decreases while the 500 nm increases. This is therefore a ratiometric sensor probe. Hyper is highly selective for H_2O_2 and cell transfection is required for its use. Hyper has been demonstrated as a valuable tool to monitor hydrogen peroxide generated in different cellular compartments (Malinouski *et al.*, 2011). The fusion Hyper-PTS1 has recently been used for specific hydrogen peroxide detection in peroxisomes as well (Gehrmann and Elsner, 2011).

New probes for hypochlorous acid

SULFONAPHTHOAMINOPHENYL FLUORESCEIN (SNAPF). SNAPF is a newly developed fluorescein-derived probe for the specific detection of intracellular levels of HOCl generated by MPO activity. In the presence of HOCl, the 4-aminophenyl function suffers oxidative cleavage to release fluorescein. This probe can be used for the *in vivo* non-invasive detection of HOCl (Freitas *et al.*, 2009).

2.8 What Should We Do to Evaluate the Effect of Antioxidant Intake on Antioxidant Capacity *In Vivo*? Some Recommendations

The *in vitro* assays described above actually reflect the reductive capacity of the polyphenolic molecules. To be considered an antioxidant with biological relevance, substances must be able to:

1. Scavenge or inhibit the production of free radicals locally generated.
2. Result in significant changes in oxidative stress biomarkers or increases in the antioxidant capacity of plasma or specific tissue.

When designing an intervention study with polyphenolic antioxidants, it is therefore recommended to include assays for total antioxidant capacity and levels of oxidative stress biomarkers. The following tips provide some recommendations for the proper assessment of the impact of consumption of antioxidants *in vivo*:

Tip 1. Always try to measure serum or plasma antioxidant capacity indexes before and after antioxidant intake (TAC; ORAC; FRAP; ABTS; CUPRAC). Express your results as Trolox equivalents.

Tip 2. It is highly recommended to measure some oxidative stress biomarkers: analysis of antioxidants in plasma and tissues. You can measure the ratio of endogenous antioxidant systems, for instance, (GSH/GSSH; UQ/UQH_2), or enzymes (CAT, SOD, GPx, GST, Prx) or vitamin E/C levels, carotenes, etc., or analysis of oxidation products (y-tocopheryl quinone, 5-nitro-y-tocopherol, allantoin, nitrotyrosine).
Tip 3. Try to measure some biomarkers of damage ascribed to ROS. Damage to lipid, DNA and protein substrates could be assessed by means of: lipids (TBARS and MDA levels, DODE, HETE, conjugated dienes, isoprostanes, oxidized LDL, oxy-sterols); DNA (specific ROS damage to DNA could be evidenced by HNE, Comet assay, thyamine glycol, 5-hydroxyadenine, 8-hydroxyguanine); and proteins (specific ROS damage to proteins: carbonyls, MPO, lipofucsin, AGEs, oxidated thiols).

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3 Proanthocyanidins in Cacao

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3.1 Introduction

Cacao or cocoa (*Theobroma cacao* L., Malvaceae, formerly Sterculiaceae) is a plant that had a single origin in the Upper Amazon near the eastern edges of the Andes and was introduced early into Central America and Mexico (Motamajor *et al.*, 2002, 2003; Bennett, 2003; Bartley, 2005). Cultivation of this plant represents one of the most sophisticated pre-Hispanic agroforestry systems (Gómez-Pompa *et al.*, 1990). The seeds of this plant had religious, ceremonial, medicinal, beverage and even monetary value for Olmecs, Mayas, Aztecs and other groups in Mesoamerica (Bennett, 2003; Schwan and Wheals, 2004).

In general, cultivars of this highly variable species fall into three categories, one of two botanical varieties or their hybrids:

- Criollo *(T. cacao* var. *cacao).* The word criollo means 'native', because this type is distributed from southern Mexico to South America, north and west of the Andes (Gómez-Pompa *et al.*, 1990). The fruits are oblong to ovoid in shape, tapering to a point, and have five or ten longitudinal ridges; seeds have yellowish white cotyledons. Some, such as the Arriba type of Western Ecuador, are considered among the finest types of cacao (Schwan and Wheals, 2004), but Arriba is considered to be a Forestero (Motamayor *et al.*, 2008). Criollo seeds contain no anthocyanins, but more caffeic acid aspartate than other cacao types (Elwers *et al.*, 2009).
- Forastero *(T. cacao* var. *sphaerocarpum).* The word forastero means 'foreign', because this type was introduced to Mesoamerica from the Amazon basin. The fruits are ellipsoid to round, lacking a pointed tip, and may be furrowed but have a smooth surface otherwise. The cotyledons are violet. Forasteros are higher yielding and more vigorous than criollos but considered to have inferior quality. About 80–90% of cacao production is based on the forastero form, owing to its superior yield, vigour and disease resistance. 'Amelonado' is the major West African cultivar and the predominant type grown worldwide. Arriba Nacional is grown in Ecuador. Several previous workers failed to find significant differences in polyphenol and

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catechin content between Criollo and Forastero types (Elwers *et al.*, 2009).

- Trinitario (hybrids of criollo and forastero forms). These hybrids, which originated in Trinidad, are sometimes classified as a subgroup of the forasteros (Gómez-Pompa *et al.*, 1990). Because they are hybrids, they are highly variable from seed, unless the seed is derived from known crosses. The seed quality is intermediate between that of the criollos and the forasteros, as are other characteristics. Several previous workers failed to find significant differences in polyphenol and catechin content among Criollo, Forastero and Trinitario types (Elwers *et al.*, 2009) This classification into subspecies and hybrids is still used in worldwide cocoa trade, but recent results of molecular studies led to the recognition of ten genetic clusters of cocao instead of two genetic groups (Motamayor *et al.*, 2008).

Phenolic compounds represent a large group of molecules widely distributed in the plant kingdom, where they have a variety of functions in growth, development and defence. Although there does not seem to be a significant difference in the amount of procyanidins between the major types of cacao, there is major seed-to-seed variation in the amounts encountered (Niemenak *et al.*, 2006); flavan-3-ols, anthocyanins and proanthocyanidins are responsible for most of the bitter and astringent flavours of chocolate products. The aroma compounds of cacao are largely due to reducing sugars, free amino acids and oligopeptides that arise during the fermentation and drying processes (Schwan and Wheals, 2004) and undergo non-enzymatic Maillard reactions during drying and roasting. The alkaloids theobromine and caffeine also contribute to bitter tastes in cacao products.

3.2 Raw Cacao Beans

Proanthocyanidins accumulate from approximately 6.9% to levels as high as 18% dry weight of seeds of *Theobroma cacao* (Elwers *et al.*, 2009; Redovniković *et al.*, 2009; Liu, 2010). Epicatechin represents 2–4% of the dry weight of defatted cocoa seed powder (Niemenak *et al.*, 2006). Other flavonoids of *Theobroma cacao* seeds include (2R,3S)-(+)-catechin, (2R,3R)-(−)-epicatechin, and the dimers epicatechin-(4β→8)-catechin (procyanidin B1), epicatechin-(4β→8)-epicatechin (procyanidin B2), epicatechin-(4β→6)-epicatechin and the trimer epicatechin-(4β→8)-epicatechin, epicatechin-(4β→8)-epicatechin (procyanidin C1) (Porter *et al.*, 1991). Three other more complex compounds, 3-*O*-β-D-galactopyranosyl *ent*-epicatechin-(2α→7, 4α→8)-epicatechin, 3-*O*-L-arabinopyranosyl *ent*-epicatechin-(2α→7, 4α→8)-epicatechin and epicatechin-(2β→5, 4β→6)-epicatechin were also isolated from fractions of the seed extracts (Porter *et al.*, 1991; Tückmantel *et al.*, 1999).

The procyanidins in cocoa were established by column chromatography, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and negative ion fast atom bombardment mass spectrometry (FAB/MS) to include oligomers through heptamers (Porter *et al.*, 1991). This work established that the structures through tetramers consisted primarily of (−)-epicatechin units. Later work (Clapperton *et al.*, 1992) established the structures through octamers with a combination of column chromatography, reversed-phase HPLC, and positive ion liquid secondary ion (LSI) mass spectrometry. In subsequent studies, the investigators used normal-phase HPLC coupled with atmospheric pressure ionization with electrospray (API-ES) mass spectrometry to measure the degree of polymerization of the seeds of *Theobroma cacao* and chocolate, and to separate and simultaneously identify groups of procyanidin oligomers (Hammerstone *et al.*, 1999). Sodium ion was also used to assist ionization of the proanthocyanidins. Monomers through decamers were observed (Wollgast *et al.*, 2001; Counet *et al.*, 2004). Oligomers from tetramers to decamers also formed multiply charged ions.

Although proanthocyanidin with epicatechin units are the major components in unprocessed cacao, (+)-catechin has been found in unfermented, dried cocoa beans, and epimerization of (−)-epicatechin

to (+)-catechin in cocoa procyanidins has been observed during heating of samples (Prior and Gu, 2005).

Minor amounts of glycosides of procyanidins, anthocyanins and flavonols also occur in cacao seeds (Sanchez-Rabaneda *et al.*, 2003). Monomeric glycosides with *m/z* 451, 737 and 707 are believed to be monomeric glycosides and doubly linked dimeric glycosides from hexose and pentose sugars, respectively (Hammerstone *et al.*, 1999). Two procyanidin glycosides related to procyanidin A2 were isolated from cacao seeds (Porter *et al.*, 1991). Quercetin 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-β-D-arabinopyranoside were also found in cacao seeds (Hammerstone *et al.*, 1999). Quercetin 3-*O*-β-D-galactopyranoside and possibly quercetin 3-*O*-rutinoside also have been reported (Elwers *et al.*, 2009). Only traces (0.02–0.4%) of these glycosides and no quercetin aglycone were found in fresh cacao seeds. Fresh cacao seeds, other than those of the Criollo type, contain purple anthocyanidin pigments, 3-*O*-β-galactosyl- and 3-*O*-α-L-arabinosylcyanidins (Niemenak *et al.*, 2006). During fermentation, these pigments are hydrolysed by glycosidases, resulting in a browning of cotyledons.

Although a long list of other minor phenolics have previously been reported to occur in extracts of cacao seed, many were not detected in recent studies. It seems probable that many of these compounds were artefacts generated during postharvest processes or during the analyses themselves (Elwers *et al.*, 2009). Little is known about the processes that determine the content of phenolic compounds in unfermented seed, but the proanthocyanidin content of various types of cacao is usually very similar. The polyphenols of raw cacao beans differ substantially, however, from those of cacao liquor, cocoa powder or chocolate, which are formed by processes involving fermentation, drying and roasting (Redovniković *et al.*, 2009).

3.3 Fermented Cacao Beans

The first stage of chocolate production consists of a natural, 7-day microbial fermentation of the pectinaceous pulp surrounding beans of the tree *Theobroma cacao* (Schwan and Wheals, 2004). A sequence of anaerobic and aerobic aeration is essential for satisfactory fermentation processes. There is a microbial succession of a wide range of yeasts, lactic-acid, and acetic-acid bacteria during which high temperatures of up to 50°C and microbial products, such as ethanol, lactic acid and acetic acid, kill the beans and cause production of flavour precursors (Schwan and Wheals, 2004).

The flavours and flavour precursors of cacao beans are developed during the primary processing of cacao beans, namely fermentation and drying. This process involves the action of various microorganisms in the cacao pulp and the action of enzymes on carbohydrates, proteins and polyphenols in the cacao beans (Luna *et al.*, 2002; Schwan and Wheals, 2004; Camu *et al.*, 2008). Peptides and amino acids are generated from storage proteins by the action of endogenous proteases (Biehl *et al.*, 1993; Voigt *et al.*, 1993). Sugars, peptides and amino acids are further modified through Maillard reactions during drying and roasting (Camu *et al.*, 2008). During the aerobic phase of fermentation, the acidification of cacao beans by acetic acid leads to the death of the embryo, which is necessary for further changes in flavour and proanthocyanidin content.

Fermentation is not only essential for the formation of chocolate flavour precursors, but autoxidation and ill-defined enzymatic processes convert the polyphenols into the characteristic brown coloration of well-fermented cacao (Porter *et al.*, 1991). During fermentation, the phenolic compounds that are originally found in vacuoles of specific cells diffuse through the cotyledon (de Brito *et al.*, 2002; Camu *et al.*, 2008). Simultaneously, the proanthocyanidins are converted through oxidation by polyphenol oxidases that promote *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (de Brito *et al.*, 2002; Luna *et al.*, 2002; Redovniković *et al.*, 2009). None the less, diminution of the phenolic compounds cannot be explained only by enzymatic oxidation because the conditions for polyphenol oxidase activity are not

appropriate during fermentation. Conditions during drying are more appropriate for this decrease (Wollgast and Anklam, 2000).

The amount of epicatechin decreases by about 90% and that of procyanidins by 10–50% during fermentation (Kim and Keeney, 1984; Camu *et al.*, 2008). The amount of catechin and of the associated dimer epicatechin-(4β→8)-catechin increases, however, during this process (Porter *et al.*, 1991). Cyanidin 3-*O*-glycosides disappear rapidly during the fermentation process (Camu *et al.*, 2008). Fully fermented cacao seeds when cut have a brown coloration, whereas inadequately fermented beans have a violet colour (Rohsius *et al.*, 2010).

Other changes in phenolic content occur during the drying process. During drying, the water content is reduced from about 45% to 7% (Camu *et al.*, 2008). Pyrazines are usually produced during the drying process. These components of the odour complement result from reactions of amino acids and reducing sugars (Jinap *et al.*, 2008). Methylpyrazine compounds constitute one of the important groups of cocoa flavours, representing about 40% of the compounds identified from the cocoa aroma fraction (Jinap *et al.*, 2008).

During fermentation and drying, the level of (–)-epicatechin decreased to 2615 mg/kg, about 8% of the level of unprocessed seed (Criollo type) (Elwers *et al.*, 2009). The level of (+)-catechin decreased to 7.2 mg/kg, about 1% of its former level. Caffeic acid aspartate is more resistant to drying and about 33% of the original amount remains (Elwers *et al.*, 2009).

There is also a decrease in the amount of phenolic compounds that cannot be explained only by enzymatic oxidation during fermentation because the conditions for polyphenol oxidase activity are not appropriate during that process. Conditions during drying are more appropriate for the loss of proanthocyanidins (Wollgast and Anklam, 2000). A decrease in the amount of these polyphenolic compounds during drying has been attributed to enzymatic browning followed by non-enzymatic browning from quinone polymerization. Additionally, polyphenols form complexes with the proteins and polysaccharides that are present (Niemenak *et al.*, 2006). The combination of these processes reduces both the solubility and astringency of the proanthocyanidins, modifying the bitterness and astringency of the seeds at this stage (Nazaruddin *et al.*, 2006).

3.4 Roasted Cacao Beans

After cacao beans are dried, they are often stored for a period of time and shipped to various places in the world where they are consumed. The processing includes the roasting of cocoa nibs at about 120–140°C for 10–20 min. In the course of roasting, up to 20% of the total phenolic compounds can be degraded.

Proteolysis in cacao beans during fermentation gives rise to amino acids and peptides (Biehl and Passern, 1982) that are responsible for the development of cocoa aroma in the subsequent roasting process. The reactions of polyphenols, sugars and amino acids are responsible for much of the flavour and colour of the roasted cacao beans (Luna *et al.*, 2002; Redovniković *et al.*, 2009). Roasting leads to the development of specific aromas via the Maillard reaction, caramelization of sugars, degradation of proteins and formation of volatile compounds such as pyrazines (Luna *et al.*, 2002). On fermentation and roasting, a major portion of the proanthocyanidins is converted to largely insoluble red-brown material resulting in the characteristic colour of chocolate (Porter *et al.*, 1991). Although the amount of polyphenols is substantially reduced by enzymatic browning during fermentation (Redovniković *et al.*, 2009), only small additional changes occur during the roasting process (14%) (Nazaruddin *et al.*, 2006; Jolić *et al.*, 2011). Maillard compounds are primarily affected by the temperature and time of roasting (Camu *et al.*, 2008).

Although proanthocyanidin with epicatechin units are the major components in unprocessed cacao, epimerization of (–)-epicatechin to (+)-catechin in cocoa procyanidins has been observed (Prior and Gu, 2005).

This may be important because cacao liquor and chocolate are manufactured by fermentation and roasting of cacao beans. In addition, (–)-catechin has been detected during roasting of cacao seeds and may also arise by epimerization during roasting (Kofink *et al.*, 2007).

3.5 Cacao Liquor

Fermented and roasted cacao is the source of cacao liquor, a major precursor of chocolate and cocoa. After roasting and winnowing (removing the outer shell from the cacao beans), the seeds are ground making cacao liquor. The heat that is generated melts the cocoa fat generating a liquor. The liquor hardens to unsweetened chocolate, often called 'baking chocolate', when it cools below 35°C. The exact composition of cacao liquors depends on the cacao variety, post-harvest processes, such as fermentation and drying, and roasting conditions (Wollgast and Anklam, 2000).

Cacao liquors of various sources differ quantitatively, but mostly have the same components, i.e. similar profiles (Redovniković *et al.*, 2009). Polyphenol levels in defatted cacao liquor are approximately 30–74 mg/g (Luna *et al.*, 2002), but a comparison of the amounts of polyphenols present in cacao products based on the literature is difficult because of differences in the methods used to determine this group of compounds. Cacao liquor consists of approximately 50% fat content (Luna *et al.*, 2002). Theobromine content varies from 17 to 26 mg/g of dry weight of the defatted material (Luna *et al.,* 2002). Caffeine normally occurs at lower levels. Sugars vary in the range of about 20–25 mg/g in cacao liquor and largely consist of sucrose, fructose and glucose, in decreasing order.

Although the amount of proanthocyanidins in cacao liquor is less than in the original seeds, the overall composition seems to be relatively similar. Mass spectral analysis of dark chocolate, which is similar to cacao liquor, in the negative mode revealed the presence of a complex series of procyanidin oligomers. The relative abundance of proanthocyanidin oligomers in chocolate extract is much lower than in extracts of non-fermented seeds, but ions for proanthocyanidins through nonamers were found, as were ions for procyanidin glycosides. Quercetin glycosides, theophylline and other compounds also were encountered (Hammerstone *et al.*, 1999).

Purification of polar fractions from cacao liquor extracts gave 17 phenolics including four new compounds. These new compounds included a *C*-glycosidic flavan, a *O*-glycoside of a dimeric and two *O*-glycosides of trimeric A-linked proanthocyanidins (Hatano *et al.*, 2002).

Procyanidin B2, protocatechuic acid, (+)-catechin, (–)-epicatechin, 3T-*O*-β-D-galactopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (T refers to the 'top' moiety of the structure as usually drawn with the epicatechin unit at the top, and extension units below), procyanidin C1, procyanidin B5, cinnamtannin A_2, 3T-*O*-α-D-Galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin, proanthocyanidin A1, proanthocyanidin A2, bis 8,8′-catechinylmethane and quercetin 3-*O*-α-L-arabinopyranoside were isolated by extraction from cacao liquor with ethyl acetate and *n*-BuOH, followed by chromatography Sephadex LH-20 and Toyopearl HW-40 and similar resins. A few additional and somewhat unusual compounds have been reported. The *O*-glycoside of a dimer and two *O*-glycosides of A-type procyanidins have been isolated from cacao liquor. However, direct evidence has been obtained that B-type procyanidin dimers convert to A-type dimers by oxidative reactions under mild conditions (Prior and Gu, 2005) and thermally catalysed reactions may be responsible for the formation of the A-type compounds.

3.6 Cocoa Proanthocyanidins

Pressure is applied to the cacao liquor (while slightly heated) to remove some of the fat, which is also called cocoa butter. The remaining cocoa solids usually contain 10–25% cocoa butter. The solids are then ground to cocoa powder. However, because

many investigators use the term 'cocoa' to indicate more than one type of product from *Theobroma cacao*, it is usually necessary to determine carefully the nature of the starting material used. For example, Hammerstone *et al.* (1999) isolated a number of compounds from 'cocoa' but in their experimental portion indicated that they used fresh seeds in their study.

The proanthocyanidin content of cocoa powder is very similar if not identical to that of cacao liquor. The ratio of flavan 3-ols to other polyphenols has, however, been found to be higher in cocoa powder than in cacao liquor or chocolate (Hammerstone *et al.*, 1999; Natsume *et al.*, 2000).

3.7 Localization of the Proanthocyanidins in Cacao Seeds

The cotyledon mesophyll of seeds of *Theobroma cacao* and related species contains mostly polysaccharides and lipid and protein reserve cells (Martini *et al.*, 2008). The seeds of *Theobroma cacao, Theobroma subincanum* and *Theobroma grandiflorum* are more than 50% lipids; the lipid globules gradually accumulate adjacent to the cell wall. The protein reserves of mature cells of *T. cacao* are densely scattered among the lipid bodies along with occasional scattered starch granules. Polyphenolic cells are scattered throughout the mesophyll, but also aligned with the respective vascular bundles. A peculiarity of mature seeds of *T. cacao* is the presence of rows of polyphenolic cells displayed perpendicular to the cotyledon mesophyll edge (Martini *et al.*, 2008) and in lines near the epidermis (Martini *et al.*, 2008; Elwers *et al.*, 2009).

The reduced content of polyphenols in Criollo cocoa is caused by faster decrease in amount of the compounds during seed processing in contrast to other cocoa types and sub-groups (Elwers *et al.*, 2009). This decrease may be responsible for the mild flavour of Criollo type products. Characteristics of the Criollo seed shell or cotyledon tissue may permit a faster invasion of pulp degradation products into the seed and to a stronger leakage of phenolic compounds during fermentation. Higher residual polyphenol oxidase and peroxidase activities may facilitate a higher oxidation of phenolic compounds in Criollo seeds during drying. The content of caffeic acid aspartate in cocoa seed seems to be determined genetically. The amount of phenolic substances in cacao seed is linked to the nutrient supply of the mother plant. When more nitrogen is present, the content of caffeic acid aspartate increases and that of polyphenolic compounds decreases. The elevated content of amides in plants is a typical result of fertilization with nitrogen.

3.8 Bioavailability of Proanthocyanidins

Many proanthocyanidins are found in cacao products. Although these compounds are consumed in reasonably large quantities by many people (100–200 mg/day), they are poorly absorbed in the upper intestine and are not degraded *in vivo* by the human stomach (Rios *et al.*, 2002; Manach *et al.*, 2004). Some of them react with protein to form insoluble complexes that are apparently excreted intact. Others are degraded by microbial action in the colon. Only catechin, epicatechin, some dimeric and to a lesser extent trimeric proanthocyanidins are found in plasma usually as the glucuronides (Manach *et al.*, 2004). Phenolics are often detoxicated by methylation, sulfonation or glucuronidation in the small intestine or later in the liver, facilitating excretion by either the urinary or biliary paths. Procyanidin B2 has been recovered from urine (Urpi-Sarda *et al.*, 2008).

Proanthocyanidins are poorly absorbed but are metabolized to a great extent by the intestinal microbiota into various phenolic acids (Urpi-Sarda *et al.*, 2008), mainly phenylpropanoic, phenylacetic and benzoic acid derivatives.

3.9 Important Health Benefits

Although proanthocyanidins are important for their organoleptic properties (mainly

bitterness and astringency) in cacao and chocolate products, they also seem to have a host of health benefits in human diets. The antioxidant activity of these compounds seems to be important in many, but not all, of these cases. For example, isolated proanthocyanidins have inhibitory effects on NADP-dependent lipid peroxidation in microsomes and on the autoxidation of linoleic acid. These effects were attributed to radical-scavenging activity in peroxidation chain reactions (Hatano *et al.*, 2002). The NADPH-dependent lipid peroxidation in rat liver microsomes was inhibited by almost all of polyphenols tested, but, of the compounds isolated, procyanidin B2 and B5 had equal and the most powerful inhibitory effect. Additionally, procyanidin B2 had the greatest radical-scavenging effect on the DPPH radical, a common measure of antioxidant activity.

Cocoa polyphenol was fractionated from commercial cocoa powder that contained 413 epicatechin-equivalent phenolics. These phenols dose-dependently inhibited xanthine oxidase activity, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide-anion generation in cultured human leukaemia HL-60 cells. The levels of cyclooxygenase (COX)-2 expression induced in mouse skin after 4-h treatment with topical TPA was also diminished significantly by pre-treating with cocoa polyphenol for 30 min. Cocoa polyphenol at the same doses inhibited TPA-induced nuclear translocation of transcription factor p65 and subsequent DNA binding of nuclear factor-κB (NF-κB) by blocking the degradation of 1-κBα in mouse skin. Moreover, phosphorylation of p38 mitogen-activated protein kinase in ICR mouse skin was suppressed by oral treatment with cocoa proanthocyanidins. Because cellular pro-inflammatory and pro-oxidant states are closely linked to tumour promotion, the antioxidant and anti-inflammatory properties of cocoa proanthocyanidins may constitute the basis of possible antitumor-promoting effects of this substance (Lee *et al.*, 2006).

A pentameric procyanidin from *Theobroma cacao* selectively inhibits growth of human breast cancer cells (Ramijak *et al.*, 2005). This compound causes G_0/G_1 cell cycle arrest in human breast cancer cells in culture by an unknown mechanism. Further studies revealed a specific dephosphorylation of several G_1-modulatory proteins without changes in gene expression (Ramijak *et al.*, 2005). Synthetically derived pentameric oligomers based on the structure of those from cacao that were formed from (–)-epicatechin units also inhibited the growth of several breast cancer cell lines. In this case it was also established that the outcome was based on the induction of cell cycle arrest in the G_0/G_1 phase. Subsequent cell death is more likely necrotic rather than apoptotic (Kozikowski *et al.*, 2003).

Chocolate products vary widely in flavonoid content. On the basis of limited data, approximately 150 mg of flavonoids in a single dose is needed to trigger a rapid antioxidant effect and changes in prostacyclin. Various types of chocolate contain between 0.09 and 4 mg procyanidins per gram. Thus, the amount of chocolate to exert acute and chronic antioxidant effects and changes in prostacyclin are between 38 and 125 g, respectively (Kris-Etherton and Keen, 2002).

Habitual chocolate users performed better in all cognitive tests and had significantly reduced risk for poor test performance in most tests. The mean intake of chocolate among users was as little as 8 g/day (Nurk *et al.*, 2009). An effect of cocoa flavonols on cerebral blood flow raises the possibility that there may be a benefit to increasing blood flow and enhancing brain function in situations where individuals are cognitively impaired (MacDonald, 2007).

In studies of the Kuna Indians of Panama, who are heavy consumers of cocoa, the Indians have a very low rate of hypertension and cardiovascular diseases (Bayard *et al.*, 2007). However, the same people living on mainland Panama, who do not drink the flavanol-rich chocolate had much higher rates of heart disease and of cancer (Hollenberg, 2007). These workers observed that improvements in blood vessel function following consumption of flavanol-rich cocoa are paralleled by an increase in the circulating pool of nitric oxide, a molecule that helps dilate blood vessels and keep them pliable.

The levels of blood glucose and fructosamine were higher in diabetic obese mice than in control mice fed a diet containing 0, 0.5 or 1% cacao liquor proanthocyanidins. The diet containing 0.5 or 1% cacao liquor proanthocyanidins in diabetic obese mice decreased the levels of blood glucose and fructosamine without significant effects on body weights or food consumption. Dietary supplementation with cacao liquor proanthocyanidins can dose-dependently prevent the development of hyperglycaemia in diabetic obese mice. The dietary intake of food or drinks produced from cacao may be beneficial in preventing the onset of type 2 diabetes mellitus (Tomaru *et al.*, 2007).

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4 Bioaccessibility and Bioavailability of Bioactive Compounds in Food and Plant Determination

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4.1 Introduction

The bioactive compounds in foods can have benefits to human health through different mechanisms. These compounds must be acquired through the diet, however, which implies that together with the bioactive compounds will be found other compounds that might interfere with nutrient absorption or availability. The food matrix is one of the most important factors to consider when studying the bioavailability of a compound. In the case of polyphenolic compounds, these are generally ingested as complex mixtures immersed in a food matrix, for example fruit or fruit juice, which then pass through the digestive process. To exert biological activities phytochemicals should be available. Therefore, it is important to determine how the digestive process can affect the bioactive compounds and their stability, which could affect their bioavailability to be absorbed.

There are two important concepts that have been widely discussed and studied: bioaccessibility and bioavailability. Bioavailability is defined as the amount of a food component that is released from the food matrix and is available for intestinal absorption after digestion (Parada and Aguilera, 2007; García-Sartal *et al.*, 2011). Alternatively, bioavailability is defined as the fraction of ingested nutrient or active substance present in foods that is absorbed and reaches the circulatory system (Brandon *et al.*, 2006; Ercan and El, 2011). Bioavailability depends on several processes, such as digestion, absorption, transport, use and disposal. Bioavailability is an important factor in nutrition because it varies for different foods, food compounds and various gastrointestinal conditions (García-Sartal *et al.*, 2011). Mechanisms that are observed in the bioavailability of a given compound are: the compound released from their matrix during digestion in the gastrointestinal tract (bioavailability); bioaccessible fraction absorption, and metabolism in bowel and liver (Brandon *et al.*, 2006).

To study the bioaccessibility and/or bioavailability *in vitro* gastrointestinal models are used in such a way to subdue the food product to various processes that occur in the digestive system. These models have been used not only for the study of bioactive compounds, but also to study the bioavailability of contaminants. The total amount of a pollutant does not always

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reflect the amount that is available in the organism; only a fraction of the contaminant could be bioavailable after oral exposure and exert their toxic action (Brandon *et al.*, 2006).

4.2 Bioaccessibility

It is widely accepted that not all constituents present in the food matrix might be completely bioaccessible; it depends on several parameters, including their initial concentration in the food matrix, matrix composition and factors related to the host, such as enzyme concentration (Bouayed *et al.*, 2012). The *in vitro* digestion methods are extremely useful to study the effects of a food matrix, conditions or interactions with other compounds, stability and properties of compounds that could affect the bioaccessibility (Bermudez-Soto *et al.*, 2007). One of the main groups of compounds under study are phenolic compounds; these compounds, because they are being recognized as beneficial to human health, have generated researchers' interest to know whether their beneficial properties are able to survive the gastrointestinal conditions that occur once ingested. Minerals have also been extensively studied, trying different food matrixes, because minerals are generally used in the fortification of food.

4.3 Phenolic Compounds

Polyphenols can exert their bioactive properties via their antioxidant properties or additional mechanisms such as those affecting intracellular signalling and gene expression. The antioxidant properties of phenolic compounds may contribute to protect health against cardiovascular disease and cancer (among others). The bioavailability of polyphenols depends on a variety of factors, including their release from the matrix during digestion (i.e. bioavailability), cell intake, metabolism and subsequent transport to the circulatory system. An important factor to consider is that during gastrointestinal digestion polyphenols might interact with other food constituents (ion chelation), could be degraded or metabolized by hydrolysis as in de-glycosylation (Bouayed *et al.*, 2012).

After *in vitro* digestion, rather than evaluating the polyphenols content, we want to know what changes have occurred with regard to antioxidant capacity. A study of different fruit juices determined that the antioxidant capacity of the bioaccessible fractions increased, whereas ascorbic acid content and polyphenols decreased by 36% and 16%, respectively. Although the content of polyphenol decreased, there was an increase in the antioxidant capacity that could be formed owing to unknown or undetected compounds with different chemical properties. It could also be possible that *in vitro* digestion releases interfering compounds that lead to overestimating the antioxidant capacity; moreover, pancreatic enzymes and bile salts are capable of binding to metals and/or trapping free radicals, which could increase the antioxidant capacity (Cilla *et al.*, 2011).

Generally, as a food is consumed, it is consumed not only as one single type but as part of a complex meal. For example, a meal could consist of a source of carbohydrates, meat and a glass of wine or juice. In the case of the polyphenols present in wine, they have chemical affinity with proteins and Fe, which can form chelates with the phenolic compounds in the lumen. A mixture of Fe and red wine after digestion has less capacity than the consumed wine antioxidant alone; this is due to the chelates Fe-phenols. Also mixing red wine with proteins (meat or casein) after digestion also decreases their antioxidant capacity (Argyri *et al.*, 2006).

The hydroxycinnamic acid more abundant in apples is chlorogenic acid, which is reduced in concentration from gastric digestion to the intestine. However, during intestinal digestion the isomers neochlorogenic acid and criptochlorogenic acid appear. Moreover, during the gastric digestion there is an increase of phloridzin and quercetin 3-o-glucoside, indicating that gastric conditions are efficient in extraction and potential availability (Bouayed *et al.*, 2012).

The bioaccessibility of anthocyanins in mulberry is greatly reduced after intestinal digestion, with recovery of only 0.34%. Anthocyanins attached to sugars make them a high molecular weight molecule and, generally, have difficulty in being absorbed. Thus, to be absorbed, they must be degraded to phenolic acids. However, it is observed, via the ability to trap free radicals, that after digestion there is a high antioxidant capacity owing to the phenolic compounds generated from the degradation of anthocyanins in the intestinal medium (Liang *et al.*, 2012). In a similar study, it was found that *in vitro* gastric simulation has no major effect on the phenolic compounds of chokeberry juice. With a pancreatin treatment, however, the anthocyanin had the greatest loss (43%); flavonols and flavan-3-ols also decreased by 26% and 19%, respectively. It is known that dietary polyphenols are highly sensitive to the alkaline conditions of the small intestine and it is believed that during digestion in the duodenum these compounds can be transformed into different structural forms, with different chemical properties (Bermudez-Soto *et al.*, 2007).

4.4 Carbohydrates

Currently, there are various studies with respect to starch digestibility that may vary by such factors as the origin and composition of starch, among others. The first enzyme involved in starch digestion is salivary amylase, which occurs in the mouth as the first stage of the digestion process. In a short time, the bolus is transported to the stomach. The gastric juice pH retards the action of amylase, but increases the starch acid hydrolysis. In the upper gastrointestinal tract, lipids bound to the starch are hydrolysed by lipases and, at this stage, an emulsion is formed that increases the area of oil–water interface, which allows a more effective enzyme action (Dona *et al.*, 2010). Likewise, studies on polysaccharides resistant to digestion by the enzymes of the small intestine show they could be delivered to the bowel in a chemically unaltered state.

Wholewheat cereal has been shown to correlate positively with reduced risk of cardiovascular disease, diabetes and certain cancers. These effects are, however, due to the effect of its fibre in the bowel microbial populations. By means of *in vitro* digestion, it was observed that wholegrain consumption increases the population of bifidobacteria, and toasted grain consumption significantly increases the growth of lactobacilli (Connolly *et al.*, 2012).

During kiwi fruit digestion *in vitro*, it was found that the insoluble fibre decreases slightly, whereas the soluble fibre increases after undergoing gastric and intestinal digestion. Furthermore, there was a reduction in molecular weight and degree of methyl esterification of pectic polysaccharides, which could have an effect on the physicochemical characteristics and could influence its functionality in the large bowel (Carnachan *et al.*, 2012). In addition, the dietary fibre konjac glucomannan (*Amophophallus konjac*), when subjected to *in vitro* digestion, has been shown to be resistant to degradation by digestive enzymes (Chiu and Stewart, 2012).

Carbonated beverages and milk are high in sugars. The food matrix in which these sugars are found have much influence on their bioaccessibility. When carbonated beverages are consumed, the bioavailability of total sugars is 55–69%, whereas for milk it is 12–85% (Choi *et al.*, 2011).

4.5 Pollutants

The main pollutants studied are metals that may be present in food. Although one can know the total content of contaminants in food, the real risk is in the bioavailability of the compound when ingested. For example, the highest concentrations of organic mercury are found in predatory fish muscles inhabiting the waters near the bottom. Through *in vitro* digestion, it was determined that between 26% and 62% of organic mercury was released into the intestinal lumen during digestion of muscle, depending on the fish species. Therefore, to declare the potential toxicity of organic mercury

content to fish in muscle may be baseless because the risk assessment should be based on the bioavailability of organic mercury (Kwasniak *et al.*, 2012). Furthermore, total arsenic bioavailability was calculated (toxic and nontoxic) in different fish and shellfish samples through *in vitro* simulation of gastrointestinal digestion, where there has been observed a high bioaccessibility (85–100%); moreover, it was observed that fish and shellfish with higher fat content have a lower arsenic bioaccessibility (Moreda-Pineiro *et al.*, 2012).

For other pollutants, fat oxidation products have been studied, which can produce toxic compounds such as the aldehydes α, β-unsaturated oxygenates (OαβUAs), especially by the oxidation of omega-6 and omega-3 acyl groups. Subjecting oils rich in these fatty acids to thermal treatment produces OαβUAs, which remain unchanged, being bioaccessible in the gastrointestinal tract and thereby able to reach the circulatory system (Goicoechea *et al.*, 2011).

4.6 Others

Flaxseed is a good source of soluble fibre and lignans, particularly the lignan secoisolariciresinol diglucoside (SDG), which can be metabolized by bowel microflora in its aglycone secoisolariciresinol (SECO) and lignans enterodiol (ED) and enterolactone (EL), resulting in health benefits through their antioxidant and phytoestrogenic activity. By simulating *in vitro* the entire digestive process, we determined that SDG was released in the large intestine and SECO was released by microbial action in the ascending colon, which then becomes lignans ED and EL forward from the transverse bowel (Eeckhaut *et al.*, 2008).

Milk-derived peptide bioactivity has been extensively studied, and opioid activity, decreased blood pressure, antimicrobial activity and immunomodulators have been reported. β-Casomorphin-7 (BCM7) is an exogenous opioid peptide derived from proteolysis of the β-casein, which was isolated from an enzymatic digestion of casein. In one study, using *in vitro* digestion determined that the formation or release of BCM7 is promoted primarily by the action of gastrointestinal proteases, independent of the milk product (De Noni and Cattaneo, 2010).

Coenzyme Q10 is a fat-soluble vitamin that plays an important role in the process of cellular breathing and energy production, and is also an important antioxidant. Coenzyme Q10 is absorbed in the small intestine. Meat, especially liver and heart, is an important source of coenzyme Q10, because of its high content in cooked meat and owing to its digestibility, which reaches up to 68% of bioaccessibility (Ercan and El, 2011).

Selenium (Se) is an essential trace element that plays an important role in the activity of enzymes involved in antioxidant protection and thyroid hormone metabolism. By consuming selenized yeast by an *in vitro* digestion it was found that selenomethionine (SeMet) was the main compound identified in the gastrointestinal extract. The results showed that 89% of total Se was extracted after gastrointestinal digestion, but only 34% was quantified as free Se (Reyes *et al.*, 2006).

Wheat grass is consumed as a food supplement in tablets because it contains essential elements such as K, Mn, Zn, Fe and Na. The bioaccessibility of these minerals was calculated through an *in vitro* gastric digestion, where 39–60% of bioaccessibility was obtained from the fresh wheat grass, whereas bioaccessibility was 17–43% in wheat grass tablets, indicating that the fresh wheat grass is an effective source of minerals (Kulkarni *et al.*, 2007).

4.7 Bioavailability

Bioavailability studies in humans have shown that many of the polyphenolic compounds vary in their absorption depending on the compound and/or food matrix. Moreover, the bioavailability of polyphenols may be diminished by a high biliary and intestinal secretion of their conjugates. Therefore, the volume of dietary polyphenols and their conjugates must remain in

the intestinal lumen and it is in the gastrointestinal tract where these compounds can have substantial benefits, such as inhibition of abnormal cell proliferation and protection against the development of cancer.

Among the methods currently used to estimate the bioavailability is the dialysis process. In the study of apple polyphenols mentioned above, also the amount of dialysable polyphenols is calculated, wherein the total dialysable polyphenols were lower than the total soluble polyphenols in the intestinal phase, indicating that the amounts of potentially available polyphenols for absorption are less than those bioaccessible in intestinal digestion, by approximately 50% (Bouayed *et al.*, 2012). But dialysis is a complex process affected by factors such as volume, composition of the buffer used, concentration of sugars in the sample or the ability of certain molecules to bind to the membrane. All these parameters can affect the dialysance of a specific compound, which cannot actually be attributed to absorption of this compound *in vivo* or levels of this compound in serum. Another method for the study of bioavailability of phenolic compounds is the use of Caco-2 cells. In a study of grape seed extract it was observed that the phenol content was not affected by gastric stimulation, but during the intestinal digestion decreased due to interaction with pancreatic proteins. Then, in the presence of Caco-2 cells, all dimers disappear, except (+)-catechin and (−)-epicatechin that decreased 44% and 85%, respectively, after 2 h of intestinal digestion. Compounds were not detected in the basal compartment of the cell monolayer (Laurent *et al.*, 2007).

β-Carotene is the most common carotenoid found in human tissue and plasma; after crossing the intestinal wall the β-carotene is converted to vitamin A and other metabolites. The bioavailability of β-carotene depends on the physicochemical state of the carotenoid in food, the type of processing/cooking, presence of other nutrients, seasonal variation and geographical origin. In carrots, carotenoids are present in semi-crystalline form or associated with proteins embedded in chromoplasts. Cooking vegetables rich in carotenoids can lead to degradation and/or isomerization of carotenoids. There are higher amounts of *trans* β-carotene on micelles in cooked carrots than in raw carrots; cooked carrots also contain higher levels of the 13-*cis* isomer and 15-*cis* isomer. All *trans* β-carotene isomers are more bioaccessible from mashed than from cooked carrots. All *trans* β-carotenes are absorbed faster than the *cis* by Caco-2 cells. Therefore, cooking the carrots increases the bioaccessibility and bioavailability of all *trans* β-carotene (Aherne *et al.*, 2010).

With respect to contaminants in marine products, it has been observed that the effect of cooking algae causes the elimination of arsenic in the cooking water. The percentages of dialysability found in raw algae are comparable to those found in cooked algae, except for sea lettuce, which yields a lower percentage when it is cooked. The percentage of dialysability in cooked algae was between 7.4 and 13.8%, which does not vary much when compared with raw algae (García-Sartal *et al.*, 2011).

4.8 The *In Vitro* Human Digestive Process

Feeding methods *in vivo*, using animals or humans, usually provide more accurate results but are long and expensive, which is why currently efforts are made in developing *in vitro* procedures. Table 4.1 shows the conditions used for various simulated gastric digestions. Enzymes commonly used in *in vitro* digestion models are pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α-amylase, lipase, bile salts and mucin. The simulation times vary according to the food matrix being studied; small particles require less digestion times than larger particles (Hur *et al.*, 2011). Generally, gastric digestion times of 1 h are used and intestinal digestion times used are 2–6 h. Models of *in vitro* digestion do not usually consider the large intestine, because compound absorption generally occurs in the small intestine (Brandon *et al.*, 2006). But there are studies that do include the processes occurring in

Table 4.1. Conditions of *in vitro* digestion.

Digestive process	Reagent	pH	Time	References
Stomach	Porcine pepsin	2.0	1 h	(Garrett, *et al.*, 1999;
Small intestine	Glicodeoxicolate	5.3–7.4	2.5 h	Aherne *et al.*, 2009, 2010)
	Taurodeoxicolate			
	Taurocolate			
	Porcine pancreatin			
	Cholesterol esterase			
Stomach	Pepsina	2.8	2 h	(Argyri *et al.*, 2009, 2011)
Small intestine	Pancreatin	6.5	2 h	
	Bile salts			
	Dialysis membrane			
Stomach	Pepsin	2.0	2 h	(Gil-Izquierdo *et al.*, 2001;
Small intestine	Pancreatin	7.5	2 h	Bermudez-Soto *et al.*,
	Bile			2007)
	Sodium cholate			
	Sodium deoxicholate			
	Dialysis membrane			
Stomach	Porcine pepsin	3.0	1 h	(Bhagavan & Chopra,
Small intestine	Porcine pancreatin	6.0–6.9	2 h	2007; Ercan & El, 2011)
	Lipase			
	Bile			
Stomach	Pepsin	2.5	30 min	(Carnachan *et al.*, 2012)
Small intestine	Pancreatin	6.5	2 h	
	Amiloglucosidasa			
Stomach	Pepsin	2.0	2 h	(Cilla *et al.*, 2008, 2011)
Small intestine	Pancreatin	6.5	2 h	
	Bile salts			
Mastication	Na_2HPO_4, KH_2PO_4, NaCl	6.75	10 min	(Gawlik-Dziki *et al.*, 2009)
	α-amylase	1.2	2 h	
Stomach	Pepsin	6.0	1 h	
Small intestine	Pancreatin			
	Bile salts			
	NaCl, KCl			
Ascending colon	Faecal inoculum, nutrient medium	5.5		(Makivuokko *et al.*, 2006;
Transverse colon	Faecal inoculum, nutrient medium	6.0		Makelainen *et al.*, 2007)
Descending colon	Faecal inoculum, nutrient medium	6.5	48 h	
Sigmoid colon	Faecal inoculum, nutrient medium	7.0		
Stomach	Chlorhidric acid	4.5–1.7	1.5 h	(Minekus *et al.*, 1995; Yoo &
Duodeno	Bile	6.5	1 day	Chen, 2006)
	Pancreatin			
	Sodium bicarbonate			
Jejunum	Sodium bicarbonate	6.8		
Ileum	Sodium bicarbonate	7.2		
Large intestine	Faecal samples	5.8	3 d	
	Nutrient medium			
	Dialysis membrane			
Stomach		2.0–2.5	2 h	(Molly *et al.*, 1993;
Small intestine	Oxgall, pancreatine, $NaHCO_3$	5.0–6.0	6 h	De Boever *et al.*, 2000;
Transverse colon	Faecal sample, carbohydrates	5.5–6.0	18 h	Possemiers *et al.*, 2004)
Descending colon	Faecal sample, carbohydrates	6.0–6.4	36 h	
Sigmoid colon	Faecal sample, carbohydrates	6.6–6.9	22 h	

the colon because other reactions occur where the intestinal microbiota are involved. Several *in vitro* gastrointestinal models have been designed for different studies. Most of the *in vitro* models study only gastric digestion and digestion of the small intestine because, by means of enzymes and different pH conditions, the compound of interest may be detected therefore allowing the determination of the bioaccessibility and/or bioavailability. Generally, for each *in vitro* incubation, while maintaining the temperature constant at 37°C, the sample is subjected to digestion in the stomach with a pH close to 2.0 and incubating with pepsin (from pork mucosa) in a bath, stirring it for 2 h. Then it is neutralized and pancreatin (from pork pancreas) and bile salts are added and incubated for 2 h (Gil-Izquierdo *et al.*, 2001).

Among the models that simulate the upper gastrointestinal tract is a system using only a bioreactor to study the passage in the stomach and intestines; this system can alter the pH and adapt it to different retention times. The simulation of the stomach and small intestine is performed in a flask with stirring, and combining gastric and acid solutions, specifically with pepsin in acid medium to simulate stomach and pancreatin and bile salts in a neutral medium for the small intestine (Sumeri *et al.*, 2008); this system is a more realistic replication of the conditions of the upper gastrointestinal tract.

Of the models that simulate the entire gastrointestinal tract is the Human Intestinal Ecosystem Simulator (SHIME), which consists of five or six bioreactors with controlled pH conditions simulating the stomach, small intestine, ascending, transverse and descending colon (Molly *et al.*, 1993; De Boever *et al.*, 2000; Possemiers *et al.*, 2004). Another model used is a computer-controlled dynamic model called TIM (TNO intestinal model), part of the Netherlands Organization for Applied Scientific Research (TNO), which consists of four chambers to simulate conditions in the stomach and small intestine by kinetic pH, bile salt concentration and transit of chyme (Minekus *et al.*, 1995). The TNO intestinal model of the stomach and small intestine is perhaps the most elaborate and allows the manipulation of many parameters, including gastric and intestinal pH regulation, pancreatic juice flow including digestive enzymes, peristalsis for the mix, transit times and continuous removal of digested compounds (Carnachan *et al.*, 2012). Both simulators, SHIME and TIM, because they are very simplified, show faults in the absorption of metabolites and fluids, and colonization of microorganisms in the bowel, among others. There are also suggestions to incorporate simulated chewing and peristalsis for a complete digestive process (Yoo and Chen, 2006). To simulate the digestive process in the colon, the simulator EnteroMix® consists of four reactors that recreate the conditions of the bowel in all segments – ascending, transverse, descending and sigmoid – using the same faecal inoculum obtained from one or more donors, which was developed to study the effects of carbohydrates fermentation in the colon microbial composition (Makivuokko *et al.*, 2006; Makelainen *et al.*, 2007).

To simulate the bioavailability of a bioactive compound, some authors use a dialysis membrane. Usually, this is a dialysis semipermeable cellulose membrane, which is added during the *in vitro* digestion of the small intestine (Bouayed *et al.*, 2011, 2012). This *in vitro* model simulates gastrointestinal digestion by subjecting the samples (in vials) to incubation in a bath with stirring for 4.5 h at 37°C at different pH values in the presence of pectic enzyme and by the fractionation of digestion via dialysis bags, which are immersed in the samples (Argyri, *et al.*, 2009). The dialysate (fraction inside the bag) consists of low molecular weight soluble compounds and the retained soluble and insoluble high molecular weight compounds. The retained compounds and dialysate are centrifuged and the supernatant removed (Kapsokefalou and Miller, 1991). This dialysability method has been considered a desirable option to calculate the bioavailability of Fe in a large number of samples. The molecular weight is presumably a significant factor that determines the absorption of Fe; for this reason predicting Fe bioavailability is based on the measurement

of dialysable Fe (Argyri *et al.*, 2009). Furthermore, this method has also been used to measure Zn bioavailability (Argyri *et al.*, 2011).

Another method for estimating bioavailability is to use Caco-2 cells, which is a quick and inexpensive method (Glahn *et al.*, 1997). These human colon cells are seeded at a density of 5 × 104 cells/cm^2 and grown for 21–25 days to obtain a cell monolayer (Aherne *et al.*, 2010). The mucus layer covering the intestinal epithelium has a major role in the absorption. The mucus is mainly composed of mucin, which is secreted by goblet cells in the epithelium. These cells can be emulated *in vitro* using HT29-MTX cells, cells that are human colon adenocarcinoma resistant to methotrexate. A co-culture of Caco-2 and HT29-MTX represents two major types of cells in the epithelium of the small intestine. When Caco-2 cells and goblet cells are cultured together they form a monolayer, with tight junctions between the two cell populations. This model has been used for studies of Fe bioavailability (Mahler *et al.*, 2009).

4.9 The *In Vitro* Digestive Process: Design in a Single Batch

The *in vitro* simulator of the digestive process in a single batch was designed to evaluate the bioaccessibility of flaxseed lignans. The upper gastrointestinal tract was simulated according to the methodology reported by Sumeri *et al.* (2008) with modifications; these changes altered the time of passage of the bolus through the stomach and through the small intestine. Moreover, instead of directly adding the sample, a corresponding bolus of food and saliva mixture was drawn. For this, artificial saliva was prepared according to Arvisenet *et al.* (2008). In this *in vitro* simulation conditions were recreated that occur during fermentation in the colon, keeping the whole process in the same bioreactor. The large intestine stage was performed according to methodology reported by Possemiers (2004) and De Boever (2000), with the difference that all steps occurring in the colon (ascending, transverse and descending colon), were performed in a single reactor. Other *in vitro* colon simulators have long fermentation times, of between 1 and 14 days (Yoo and Chen, 2006), which is not suitable to estimate the bioavailability, but this involves shorter fermentation times in the colon. Figure 4.1 shows a diagram of the *in vitro* simulation conditions of the entire digestive process.

An example of the use of *in vitro* digestion process in a single batch was the complete digestion process of Gouda cheese, which contained 15 g linseed meal per kg of cheese, resulting in the detection of several lignans (Fig. 4.2). During *in vitro* digestion of the small intestine secoisolariciresinol diglucoside (SDG) was identified; this plant lignan was probably released by pancreatic enzymes in the intestine. Eeckhaut (2008)

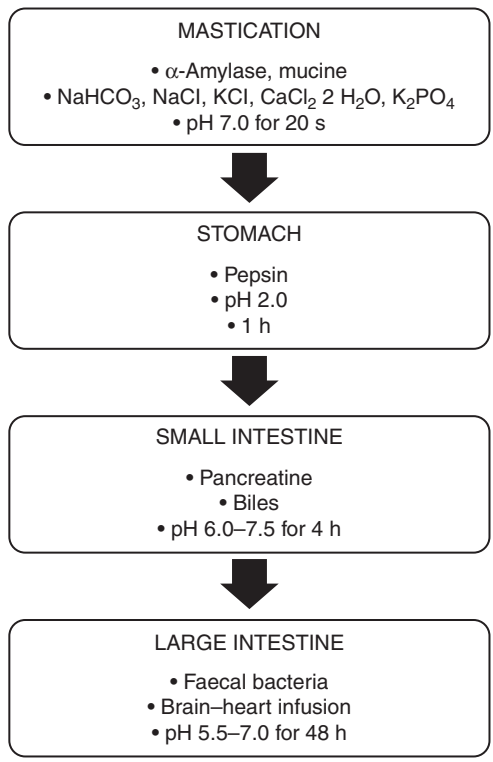

Fig. 4.1. A scheme of the digestive process *in vitro* in a single batch.

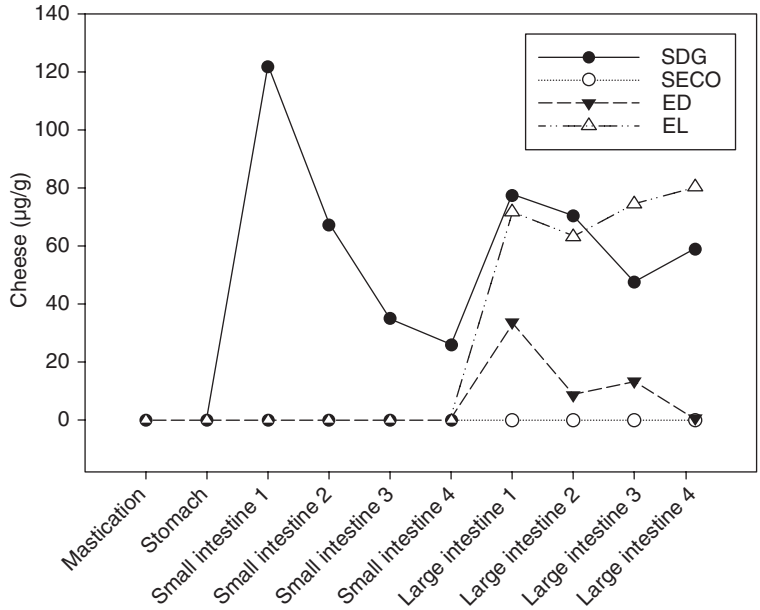

Fig. 4.2. The profile of lignans secoisolariciresinol diglucoside (SDG), aglycone secoisolariciresinol (SECO), enterodiol (ED) and enterolactone (EL) during *in vitro* digestion of cheese fortified with flaxseed.

detected no lignan in the small intestine, indicating that lactic acid bacteria present in cheese promoted its release. By the action of intestinal bacteria, SDG is metabolized to enterodiol (ED) and enterolactone (EL); however aglycone secoisolariciresinol (SECO) was not detected, probably because it is fast metabolized into ED. In Fig. 4.2, it can be observed that the SDG and ED content decreases along the colon *in vitro* digestive process and, at the same time, the content of EL is increased. The bioavailability calculated for lignans SDG, ED and EL was 1.59%, 0.99% and 2.42%, respectively.

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5 Biocidal Metabolites from Endophytes that Occur in Medicinal Plants

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5.1 Introduction

Endophytes are microbes (fungi or bacteria) that live within the plant tissues without causing any noticeable symptoms of disease (Tejesvi *et al.*, 2007). Mycoendophytes have been found in healthy tissues of all the plant taxa studied to date. Stierle *et al.* (1993) discovered the 'goldmine' bioactive compound paclitaxel (taxol) from the endophytic fungus *Taxomyces andreanae* isolated from *Taxus brevifolia. T. brevifolia* is a member of family Taxaceae and native to the north-western USA. The taxol, a natural product of *T. brevifolia,* has been used in the treatment of cancer. The isolation of taxol from *Pestalotiopsis microspora*, an endophyte of *Taxus wallichiana*, and the phytohormone gibberellin from *Gibberella fujikuroi* in rice plants, underline the potential of endophytes as a source of useful metabolites (Gehlot *et al.*, 2008). Subsequently, several scientists have studied fungal endophytes as potential source of novel and biologically active compounds.

Endophytes invade the tissues of living plants and reside in the tissues between living plant cells (Vanessa and Christopher, 2004). Some endophytes form a mutually beneficial relationship (symbiosis) with the host plants, whereas others are opportunistic pathogens. Petrini *et al.* (1992) reported that there may be more than one type of mycoendophyte present in a single plant. For example, 13 taxa of mycoendophyte were isolated from the leaf, stem and root tissues of *Catharanthus roseus* (Kharwar *et al.*, 2008). Zhao *et al.* (2011) reported that mycoendophytes are fungi that expend the whole or part of their life cycle intercellularly and/or intracellularly colonizing healthy plant tissues, causing no apparent symptoms of disease. Petrini (1991) predicted that there are more than one million fungal endophytes existing in nature. Plant endophytic fungi have been recognized as an important and novel resource of natural bioactive products with potential applications in agriculture, medicine and the food industry (Strobel *et al.*, 2004; Gunatilaka, 2006; Verma, 2009).

Herre *et al.* (2007) demonstrated that mycoendophytes play a potentially important mutualistic role by augmenting the host defence response against pathogens. Endophytes may contribute to host protection by increasing the expression of intrinsic host defence mechanisms and/or providing additional sources of

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defence extrinsic to those of the host. There has been immense interest in the prospect of these microbial endophytes as a source of novel bioactive natural products. Endophytes do show much chemical diversity: alkaloids, peptides, steroids, terpenoids, isocoumarins, quinones, phenylpropanoids, lignans, phenols, phenolic acids, aliphatic compounds, lactones and other secondary metabolites. The screening of microbial culture filtrates for the presence of secondary metabolites is an established method for the identification of biologically active molecules (Hamayun *et al.*, 2009).

Zhou *et al.* (2010) summarized the recent advances in taxol-producing endophytic fungi from all over the world. Karsten *et al.* (2007) reported herbicidal and algaecidal activity in the ethyl acetate extract of an endophytic *Phoma* sp. isolated from *Fagonia cretica*; *F. cretica* is used against fever, thirst, vomiting, dysentery, asthma, urinary discharges, liver trouble, dropsy, delirium, typhoid, toothache, stomach troubles and skin diseases. Randa *et al.* (2010) isolated a mycoendophyte (*Botryosphaeria rhodina*) from the stem of the medicinal plant *Bidens pilosa* (Asteraceae) that showed anti-inflammatory, antiseptic and antifungal effects. *B. pilosa* is used as a medicinal plant in many regions of Africa, Asia and tropical America. The extract of *B. rhodina* also had significant cytotoxic and antiproliferative effects against several cancer cell lines.

Some endophytic fungi synthesize the same bioactive compounds as their host plants (Zhao *et al.*, 2011). This finding has promoted research in secondary metabolism of endophytes from medicinal plants. In this chapter we discuss the endophytic fungi, bioactive compounds and their different biocidal activity.

5.2 Mycoendophytes and their Different Biocidal Activity

5.2.1 Mycoendophytes and anticancerous activity

Cancer is major public health problem and a leading cause of death worldwide. There were 7.6 million deaths caused by cancer worldwide in 2008 (13% of all deaths). In 2004 the number of deaths caused by cancer was 7.4 million and the cases increased by 5 million in 3 years. Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030 (Globocan, 2008). One of the major drawbacks in the usage of an anticancer agent to treat malignant diseases is its adverse toxicity. To deal with the problem, many research studies have focused on new, effective and more selective anticancer agents. Since the discovery that the world's first billion-dollar anticancer compound paclitaxel (Taxol) could be biosynthesized by *Pestalotiopsis microspora*, an endophytic fungus that colonizes the Himalayan yew tree, interest in researchers for fungal medicinal potential has grown tremendously. Hazalin *et al.* (2009) reported cytotoxicity of fungal extracts from *P. microspora* against P388 and K562 cell lines. The extracts were also more effective against P388 than the K562 cell line. Moreover, nearly half of the extracts showed activity against P388 and 25% were active against K562 cell lines. Li *et al.* (2009) investigated the anticancer activity of the endophytic fungus FSN006 isolated from the inner bark of *Juglans mandshurica*. A medium extract of FSN006 showed anti-tumour activity against the liver cancer cells HepG2 that was higher than that of curcumine. Lu *et al.* (2012) investigated the anti-tumour activity of broths from the endophytic fungi *Acremonium furcatum, Cylindrocarpon pauciseptatum, Trichoderma citrinoviride, Paecilomyces marquandii* and *Chaetomium globosum* isolated from the Chinese medicinal plant *Actinidia macrosperma*. Of the broths, 82.4% displayed growth inhibition with IC_{50} <100 μg/ml. A crude extract of *Phoma* sp. isolated from *Cinnamomum mollissimum* was assayed for cytotoxicity against P388 murine leukaemic cells and inhibition of bacterial and fungal pathogens (Santiago *et al.*, 2012). 5-Hydroxyramulosin, a polyketide compound identified in the crude extract, inhibited the fungal growth of *Aspergillus niger* (IC_{50} = 1.56 μg/ml) and was cytotoxic against murine leukaemia cells

(IC_{50} = 2.10 µg/ml). Mohana *et al.* (2012) investigated a *Fusarium proliferatum* strain endophytic of *Dysoxylum binectariferum* Hook. f (Meliaceae). The strain produces rohitukine, which showed cytotoxicity against the human cell lines HCT-116 and MCF-7 (IC_{50} = 10 µg/ml).

5.2.2 Mycoendophytes and antituberculosis activity

An increasing incidence of deaths due to tuberculosis and the known drawbacks of the current existing drugs, including the emergence of multidrug-resistant strains, have led to a renewed interest in the discovery of new antitubercular agents. Tuberculosis is a chronic infectious disease and one of the major enemies of humanity from ancient times. Today, it still remains one of the most serious medical and social problems. It is responsible for 3 million deaths per year and around 8 million cases of first-recorded disease (Rogoza *et al.,* 2011). The advances in the chemotherapy of tuberculosis in the mid-20th century have recently given way to anxiety over the evolution of drug resistance based on the genetically fixed mutations of *Mycobacterium tuberculosis* (Copp and Pearce, 2007). Moreover, nearly all drugs used for the treatment of tuberculosis and possessing different mechanisms of activity are able to cause adverse side effects in humans. It is therefore extremely important to search for new, low-toxic substances superior to the available drugs in their activity and efficiency. Metabolites synthesized by endophytic strains of *Phomopsis*, 3-nitropropionic acid and phomoxanthone, exhibited a minimum inhibitory concentration (MIC) of 0.4 µg/ml and 0.5 µg/ml on *M. tuberculosis* H37Ra, respectively (Bastian *et al.*, 2000).

Tuberculosis infections caused by different species of mycobacteria are continuously increasing. Gordien *et al.* (2010) screened the antimicrobial activity of extracts from Scottish plants, lichens and mycoendophytes on *Mycobacterium aurum* and *M. tuberculosis*. Extracts of *Juniperus communis* roots, the lichen *Cladonia arbuscula* and a mycoendophyte isolated from *Vaccinium myrtillus* showed the greatest activity on *M. aurum* (Gordien *et al.*, 2010). It is clear that mycoendophytes serve as a source of potentially useful medicinal compounds.

5.2.3 Mycoendophytes and antimalarial activity

Malaria is a disease caused by a single cell of the obligate intracellular parasite *Plasmodium* and is a major cause of mortality and morbidity worldwide. These parasites have a complex life cycle in their mosquito vector and vertebrate hosts. The primary factors contributing to the resurgence of malaria are the appearance of drug-resistant strains of the parasite, the spread of insecticide-resistant strains of the mosquito and the lack of licensed malaria vaccines of proven efficacy. *Plasmodium falciparum* is the most dangerous species for humans because it can cause acute infection leading to kidney and liver failure, coma and even death. This parasite is transmitted to humans by the female *Anopheles* mosquito. Chloroquine is the most common antimalarial drug because it is easily obtained, cheap and has few side effects. Now chloroquine is the first-line drug for malaria treatment without any complications. However, *P. falciparum* developed resistance to chloroquine and other antimalarial drugs. Endophytic fungi are a promising source of novel therapeutic agents and are of particular interest in the treatment of leishmaniasis and malaria. Martínez-Luis *et al.* (2011) reported that *Stenocarpella* sp. (strain F0275), *Nectria* sp. (strain F1491), and *Mycosphaerella* sp. (strain F2140) inhibited more than 90% the proliferation of *P. falciparum* with respect to a control. Other endophytes also showed antimalarial activity, e.g. *Xylaria* spp. found in dead wood. One of its species was isolated from *Siparuna* sp. (Jimenez-Romero *et al.*, 2008). Srinuan *et al.* (2007) reported antimalarial activity against *P. falciparum* of two novel benzoquinone metabolites, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and xylariaquinone, isolated from *Xylaria* sp. Elfita *et al.* (2011) isolated two

alkaloids, 7- hydroxy-3,4,5-trimethyl-6-on-2,3,4,6-tetrahydroisoquinoline-8-carboxylic acid and 2,5-dihydroxy-1-(hydroxymethyl) pyridin-4-on, from endophytic fungi of brotowali and studied their activity against *P. falciparum*.

5.2.4 Mycoendophytes with antifungal and antibacterial activity

Endophytes have been proven to mimic their host plant in the production of bioactive molecules, which can be of enormous pharmaceutical use (Table 5.1). A large number of anticancer drugs, antimicrobials, immunomodulators and many other useful medicinal molecules have been isolated from these endophytes. These microbes can be a suitable alternative source of the bioactive molecules usually isolated from their hosts, some of which are on the verge of extinction, and can also help in the conservation of such endangered plant species. Traditional medicinal use of plants in the cure of ailments is one of the important criteria behind the selection of hosts for isolation of endophytic fungi. So far, many antimicrobial compounds have been isolated from endophytes belonging to several structural classes such as alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids (Yu *et al.*, 2010). The discovery of novel antimicrobial metabolites from endophytes is also an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens. The insufficient number of effective antibiotics against diverse bacterial species and few new antimicrobial agents in development is probably due to relatively unfavourable returns on investment.

Santiago *et al.* (2012) reported that an endophytic fungus isolated from the plant *Cinnamomum mollissimum* showed antifungal activity against *A. niger* and also anticancerous activity. Budhiraja *et al.* (2012) isolated *Aspergillus* and *Penicillium* spp. from *Gloriosa superba*. They studied the antimicrobial activity of these endophytes against seven standard pathogenic strains: *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Saccahromyces cerevisiae* and *Candida albicans*. Vaz *et al.* (2012) isolated an endophyte *Colletotrichum* from leaves of *Myrciaria floribunda* and *Alchornea castaneifolia*, and an endophyte *Mycosphaerella* from *Eugenia* aff. *bimarginata*. The authors also screened fungal endophytes for antimicrobial activity against pathogenic microorganisms. They reported that 38 fungal extracts demonstrated antimicrobial activity against at least one of the different target microorganisms tested. *Emericellopsis donezkii* and *Colletotrichum gloesporioides* showed the best MIC values, which were lower or similar to MICs of known antibacterial and antifungal drugs. Vieira *et al.* (2012) reported diversity and antimicrobial activity of endophytic fungi isolated from *Solanum cernuum* Vell. They revealed that the most abundant taxa were closely related to *Arthrobotrys foliicola, Colletotrichum gloeosporioides, Colletotrichum* sp. *Coprinellus radian, Glomerella acutata, Diatrypella frostii, Mucor* sp., *Phoma glomerata, Phoma moricola, Phlebia subserialis* and *Phanerochaete sordid*. In total 265 fungal extracts were screened and 64 (26.01%) exhibited antifungal and antibacterial activities. Gond *et al.* (2012) isolated an endophytic fungus from *Nyctanthes arbor-tristis* and evaluated its antimicrobial activity. The endophytic *Nigrospora oryzae* showed maximum inhibition against *Shigella* sp. and *Pseudomonas aeruginosa; Colletotrichum dematium* and *Chaetomium globosum* exhibited a broad range of antibacterial activity, including inhibition of *Shigella flexnii, Shigella boydii, Salmonella enteritidis, Salmonella paratyphi*, and *P. aeruginosa*. The endophytic *C. dematium* inhibited 55.87% of the radial growth of the phytopathogen *Curvularia lunata*. The authors suggested that antimicrobial activity of these endophytic microorganisms could be exploited in the biotechnological, medicinal and agricultural industries. Lu *et al.* (2000) isolated an endophytic *Colletotrichum* species from *Artemisia annua,* which is a traditional Chinese herb, well recognized for its synthesis of artemisinin (an antimalarial drug). Further, they

Table 5.1. Diversity and antimicrobial activity of endophytes associated with medicinal plants.

No	Endophytes	Host	Antimicrobial activity	Reference
1	*Colletotrichum gloeosporioides, Guignardia* sp., *Phomopsis* sp.	*Spondias mombin*	Actinomycetes, *Escherichia coli, Staphylococcus aureus, Saccharomyces cerevisiae, Geotrichum* sp., *Penicillium canadensis, Cladosporium elatum, Mycotypha* sp.	Rodrigues *et al*., 2000
2	*Colletotrichum* species	*Artemisia annua*	*Bacillus subtilis, S. aureus, Sarcina lutea* and *Pseudomonas* sp., *Candida albicans* and *Aspergillus niger, Gaeumannomyces graminis* var. *tritici, Rhizoctonia cerealis, Helminthosporium sativum* and *Phytophthora capisici*.	Lu *et al*., 2000
3	*Colletotrichum gloeosporioides*	*Artemisia annua*	*B. subtilis, S. aureus*, and *Sarcina lutea, Helminthosporium sativum*	Zou *et al*., 2000
4	*Muscodor albus*	*Cinnamomum zeylanicum*	*Pythium ultimum, Phytophthora cinnamomi, Rhizoctonia solani, Ustilago hordei, Stagnospora nodorum, Sclerotinia sclerotiorum, Aspergillus fumigatus, Fusarium solani, Verticillum dahliae, Cercospora beticola, Tapesia yallundae, Xylaria* sp., *Muscodor albus, C. albicans, E. coli, S. aureus, Micrococcus luteus, B. subtilis*	Strobel *et al*., 2001
5	*Botryosphaeria rhodina, Calcarisporium* sp., *Fusarium* sp.	*Dracaena cambodiana, Aquilaria sinensis*	*B. subtilis, S. aureus, A. fumigatus, Cryptococcus neoformans, C. albicans*	Gong and Guo, 2009
6	*Cylindrocarpon* sp., *Phoma* sp., *Fusarium* sp.	*Saussurea involucrata*	*E. coli, B. subtilis, S. aureus, C. albicans, C. neoformans, A. fumigatus*	Lv *et al*., 2010
7	*Botryosphaeria rhodina*	*Bidens pilosa*	*Sporobolomyces salmonicolor, S. cerevisiae, C. albicans, Penicillium notatum. P. avellanea, Aspergillus terreus, Fusarium oxysporum*	Abdou *et al*., 2010
8	*Nigrospora oryzae*	*Nyctanthes arbor-tristis*	*Shigella* sp., *Pseudomonas aeruginosa*	Gond *et al*., 2012
9	*Colletotrichum gloeosporioides, Glomerella acutata, Diatrypella frostii, Phoma glomerata*	*Solanum cernuum*	Antifungal and antibacterial activity	Vieira *et al*., 2012
10	*Colletotrichum* sp.	*Myrciaria floribunda*	Antimicrobial activity	Vaz *et al*., 2012
11	*Aspergillus* sp., *Penicillium* sp.	*Gloriosa superba*	*S. aureus, B. subtilis, E. coli, P. aeruginosa, C. albicans*	Budhiraja *et al*., 2012

characterized three new antimicrobial metabolites from the culture of *Colletotrichum* species isolated from *A. annua*. Guo *et al*. (2008) also studied the new antimicrobial metabolites isolated and extracted from the culture of *Colletotrichum* species recovered from *A. annua*. These metabolites demonstrated activity against fungi and bacteria.

Rodrigues and Samuels (1999) reported the endophytic fungi *Colletotrichum gloeosporioides,* and *Guignardia* and *Phomopsis* species from leaf blades and bark of *Spondias mombin* collected from Pará and Rio de Janeiro states of Brazil. Further, they studied the antimicrobial activity of extract of *Guignardia* sp., *Phomopsis* sp. and *Pestalotiopsis guepinii* against pathogenic bacteria. Rodrigues *et al.* (2000) reported endophytic *Guignardia* and *Phomopsis* species and *Pestalotiopsis guepinii* isolated from *S. mombin*. They screened for compounds with antimicrobial activity on actinomycetes, Gram-negative, Gram-positive bacteria, yeast and filamentous fungi. Lv *et al.* (2010) isolated endophytic species of *Cylindrocarpon, Phoma* and *Fusarium* from *Saussurea involucrata*. They also studied antimicrobial activity of these fungi against pathogenic microorganisms. Guimaraes *et al.* (2008) recovered endophytes from *Viguiera arenaria* and *Tithonia diversifolia*. Moreover, they screened their antimicrobial, antiparasitic and anti-tumour activity.

5.2.5 Mycoendophytes and antioxidant activity

Antioxidant compounds in food play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce the risk of chronic diseases including cancer and heart disease. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free-radical intermediates and inhibit other oxidation reactions. Naturally occurring antioxidant compounds are commonly found in vegetables, fruits and medicinal plants. It has been observed, however, that endophytes are also a potential source of novel natural antioxidants. Tejesvi *et al.* (2008) isolated endophytic *Pestalotiopsis* species from *Terminalia arjuna, Terminalia chebula, Azadirachta indica* and *Holarrhena antidysenterica*. Furthermore, they studied antioxidant and antihypertensive activity by measuring 1, 1-diphenyl-2-picrylhydrazyl inhibitory activity, lipid peroxidation and angiotensin-converting enzyme inhibition activity. Endophytic *Xylaria* sp. isolated from the medicinal plant *Ginkgo biloba* contains compounds showing antioxidant activities (Liu *et al.*, 2007). Kajula *et al.* (2010) studied the extracellular production of siderophore and antibacterial and antioxidant compounds by endophytic fungi of Scots pine (*Pinus sylvestris* L.) and Labrador tea (*Rhododendron tomentosum* Harmaja). Huang *et al.* (2007) observed that the antioxidant capacities of the endophytic fungal cultures were correlated with their total phenolic contents, and suggested that phenolics were also the major antioxidant constituents of the endophytes. They also suggested that some of the endophytes were found to produce metabolites possessing strong antioxidant activities. The authors concluded that the metabolites produced by a wide diversity of endophytic fungi in culture can be a potential source of novel natural antioxidants.

5.3 Future Perspectives

Some endophytes produce phytochemicals that were originally thought of as characteristic of the host plant. Genetic interaction or horizontal gene transfer between the endophyte and the host has occurred over evolutionary time (Tan and Zou, 2001). This concept was proposed to explain why *Taxomyces andreanae* produces taxol. The cultured endophytes can be induced to produce the same rare and important bioactive compounds as when associated with their host plants. This would reduce the need to harvest slow-growing and possibly rare plants. It would also help to preserve the world's ever-diminishing biodiversity. Furthermore, a microbial source of a high-value product is

an economical way to produce a metabolite in a bulk quantity and thereby reduce its market price. It is also interesting to note that, as previously mentioned, some endophytes have been shown to produce new molecules with prominent bioactivity on noxious microorganisms with impact on human health and agricultural and industrial activities. This situation will also stimulate the research on endophytes in the coming years.

5.4 Conclusions

The need for new bioactive metabolites to overcome the growing problems of drug resistance in microorganisms and the appearance of new diseases is of increasing importance. The capability of fungi to produce bioactive metabolites has encouraged researchers to isolate and screen fungi from diverse habitats and environments. Endophytes are an outstanding source of both novel and bioactive natural products, which have an enormous potential for the development of new drugs and agricultural products. Consequently, endophytes are known to be a rich and reliable source of biologically active compounds with potential benefits in medicine, industry and agriculture. In contrast, the development of techniques such as combinatorial chemistry and equipment such as peptide synthesizers gives rise to exciting opportunities and expectations for the synthesis of biologically active compounds. Additionally, because most endophyte research has been conducted on cultivated species, the diversity of studied endophytes has been further restricted by limitations in the ability to cultivate endophytes in the laboratory. Research focusing on endophytes is a promising field in the chemistry and biological properties of natural products.

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6 Antioxidant Properties of Singlet Oxygen Suppressors

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6.1 Introduction

Molecular oxygen is unusual amongst common molecules in having a triplet ground state. The two electronically excited states immediately above the ground state are both singlet states: one, designated $^1\Sigma_g^+$ state, has a very short lifetime (less than a picosecond), and rapidly decays to the lower singlet state, designated $^1\Delta_g$ state, which has a lifetime of a few microseconds in water and is the main species reacting in the biological environment. Singlet oxygen ($^1O_2$) is an extremely reactive electrophilic species. It attacks with great efficiency highly alkylated double bonds, inducing damage in membranes, proteins and DNA. Its overproduction can lead to a degenerative disease called porphyria or to tumours and other human diseases in the process called photodynamic therapy.

Damage in the membranes can be initiated by the formation of lipid hydroperoxides, which can progress to cause membrane peroxidation and end up causing premature photo-ageing in the skin. The side groups of several amino acids are susceptible to oxidization by singlet oxygen, causing changes in the structure of the proteins and loss of activity, affecting, for example, the efficiency of photosynthesis.

Nature looks for strategies to protect itself from these inconveniences. For protection against sunlight, humans have melanin to avoid light from reaching proliferative cells in the epidermis. Plants, on the other hand, make use of their secondary metabolites in an appropriate and necessary way to suppress $^1O_2$. Countless antioxidants, such as flavonoids, anthocyanins, ascorbic acid, carotenoids, lycopene, tannins and vitamin A, have been described to suppress $^1O_2$. Many of these substances are synthesized from the phytoene or shikimic acid pathway, which is not present in animals. Therefore, when humans ingest these substances they benefit from their protection activities. We have organized this chapter into five topics that are: **Redox misbalance**, discussing the interaction of light of different wavelengths with different organisms inducing the formation of reactive oxygen species with an emphasis on singlet oxygen; **Singlet oxygen: general properties, detection methods and biological roles**, discussing important aspects of the chemical/biochemical properties, generation and detection of singlet oxygen (damage to proteins of the photosynthetic apparatus and membranes in general will be discussed in detail); **Chemical reactivity**, describing the main reaction routes of

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singlet oxygen, mainly involving addition to the double bonds and describing the general chemical damage caused in biomolecules; **Deactivation of singlet oxygen: kinetics and mechanisms**, describing the operational kinetics and mechanisms of singlet oxygen suppression; **Natural defences and suppressors of singlet oxygen**, unravelling properties of singlet oxygen suppressors, establishing structure/activity relationships for several classes of molecules in nature, including carotenoids, catechins, flavonoids, lycopene and tannins.

6.2 Redox Misbalance

Since the initial observation of free radicals in biological materials more than 50 years ago, the generation of several reactive oxygen species (ROS) and nitrogen species (NOS) have been proved to occur in different conditions, concentrations and exerting diverse roles (Commoner *et al.*, 1954; Augusto *et al.*, 2002; Halliwell *et al.*, 2009). Free radicals have been related to cell death, cancer, ageing and diverse degenerative processes (Harman, 1981; Beckman and Ames, 1998). The discovery of antioxidant enzymes and molecules (McCord and Fridovic, 1969; Beckman and Ames, 1998; Halliwell, 2009), and their roles in signalling and defence, brought into perspective the beneficial aspects of free radicals. The general view today is that living organisms not only have learnt to survive the chemical hazards presented by ROS and NOS but also have learnt to take advantage of the diversity of reactivities offered by them (McCord and Fridovic, 1969; Halliwell, 2009). There are several enzymatic reactions that lead to the production of free radicals including the electron transport chain in mitochondria, which is a major and continuous source of ROS (Kowaltowski and Vercesi, 1999). In a sense it is difficult to think of life without thinking of free radicals.

Redox homeostasis is kept in a steady state owing to a balance between the rate of formation of ROS and NOS and the rate of their suppression. However, the rate of free-radical production may surpass the rate of their suppression and a situation of redox misbalance is obtained (Fig. 6.1), which is implicated in a series of physiological and pathological conditions in living organisms.

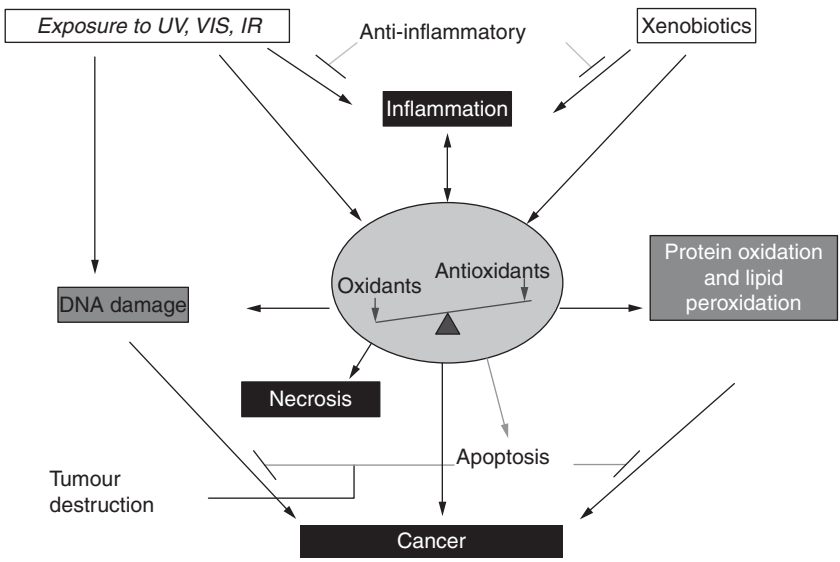


Fig. 6.1. Oxidation misbalance caused by sun exposure and/or a xenobiotic and its effect in eukaryotic cells.

In eukaryotic cells, depending on the degree of misbalance, cells can die directly by necrosis or, if there is damage in DNA, the misbalance can lead to cancer. Cells have defence mechanisms that can avoid cancer in which cells die in a controlled way by apoptosis (Simon *et al.*, 2000). Another mechanism of cellular defence, i.e. inflammation, can worsen the scenario by increasing the oxidative misbalance. Depending on the cell type, the details of the effect caused by the redox misbalance may change but the general perspective of the processes are the same.

Much work has been done in identifying the main free radicals and other ROS and NOS generated in biological environments, understanding the fine regulations of this balance, and identifying their signalling targets and the molecules and macromolecules that could help to keep the homeostasis by reacting and neutralizing ROS and NOS (McCord and Fridovic, 1969; Halliwell *et al.*, 1992). Among the ROS, singlet oxygen ($^1O_2$) has received lots of attention recently because its presence in several physiological and pathological conditions was undoubtedly demonstrated. This chapter therefore focuses on describing some of the properties and roles of $^1O_2$ in nature and technology, as well as describing the molecules that are known to suppress or decrease its lifetime and consequently its effect, and describing the mechanisms of suppression that are involved.

6.3 Singlet Oxygen: General Properties, Detection Methods and Biological Roles

Molecular oxygen is unusual amongst common molecules because it has an electronic configuration that has two highest energy electrons unpaired in the degenerated highest occupied molecular orbitals (HOMOs) (Fig. 6.2). The ground-state oxygen is therefore a triplet state. We may expect this species to have radical character and to react readily with any radical species and therefore to facilitate the progression of radical chain reactions (McCord and Fridovic, 1969; Halliwell, 2009).

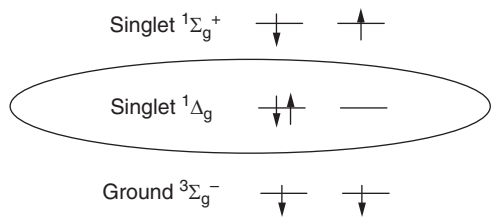


Fig. 6.2. Simplified electronic configuration of oxygen in the triplet ground $^3\Sigma_g^+$ and singlet states, $^1\Delta_g$ / $^1\Sigma_g^+$.

The two electronically excited states immediately above the ground state are both singlet states, which are generically called singlet oxygen ($^1O_2$). The first, designated sigma state ($^1\Sigma_g^+$), has a very short lifetime (less than a picosecond) and rapidly decays to the lower singlet state (Schmidt and Bodesheim, 1994); the other designated delta state ($^1\Delta_g$) has a lifetime varying from microseconds to milliseconds in the condensed phase (Schmidt, 2006). Singlet oxygen $^1\Sigma_g^+$ lifetime in solution is so short that, for practical purposes, it is deactivated immediately to the $^1\Delta_g$ state. $^1O_2$ ($^1\Sigma_g^+$) and ($^1\Delta_g$) are, respectively, 96 kJ mol^{-1} (22.4 kcal mol^{-1}) and 159.6 kJ mol^{-1} (38.2 kcal mol^{-1}), above the ground-state oxygen and, although they are not directly accessible in a spin-allowed transition from ground state, it can be trivially formed by photosensitization by triplet states. The triplet state of photosensitizers, with energy higher than 96 kJ/mol, can efficiently transfer energy to molecular ground-state oxygen forming ground-state photosensitizers and excited-state oxygen (Abdel-Shafi and Wilkinson, 2002), as detailed below. Living organisms use several molecules that absorb UV–VIS photons; therefore, photosensitization is a frequent way in which singlet oxygen is generated in living organisms. Because $^1O_2$ has an empty orbital, it can directly react with double bonds, engaging in chemical reactions that were not allowed with ground-state oxygen. Consequently, many biological molecules and macromolecules have evolved to protect living organisms from forming singlet oxygen and from its effect after it has been

formed. Singlet oxygen can also be formed by chemical/biochemical reactions, especially by the Russell Mechanism present in peroxidation processes (Miyamoto *et al.*, 2007), which may indicate a possible role for singlet oxygen in signalling events mainly related to the cellular stress response (Klotz *et al.*, 2003).

A small fraction of the population of singlet oxygen molecules that is formed decays to the ground state emitting light in the near infrared region (NIR) $\lambda_{MAX} = 1268$ nm (Krasnovskii, 1976; Khan and Kasha, 1979; Wilkinson *et al.*, 1993) and this is the spectral fingerprint of singlet oxygen molecules (Fig. 6.3). This detection method has been used to observe singlet oxygen *in vivo* in tissues and *in vitro* in different types of solutions and/or suspensions (Niedre *et al.*, 2002; Kuimova *et al.*, 2009). Usual detection equipment includes a laser system to provide light excitation and generation of singlet oxygen and a NIR fluorometer to detect its characteristic emission (Fig. 6.3). Singlet oxygen can also be detected and quantified using chemical trap methods and extremely selective probes have been designed. Natural molecules, such as betanidines found in beetroot, have also been shown to work well as a probe to detect and quantify $^1O_2$ (Bonacin *et al.*, 2009).

Singlet oxygen can be generated in a controlled and reproducible way either by chemical or by physical methods. The most used chemical methods are: the reaction between hydrogen peroxide and sodium hypochlorite, N-chlorosuccinimide and alkaline hydrogen peroxide; and the thermolysis of several endoperoxides (Baptista, 1998). By physical methods, it is possible

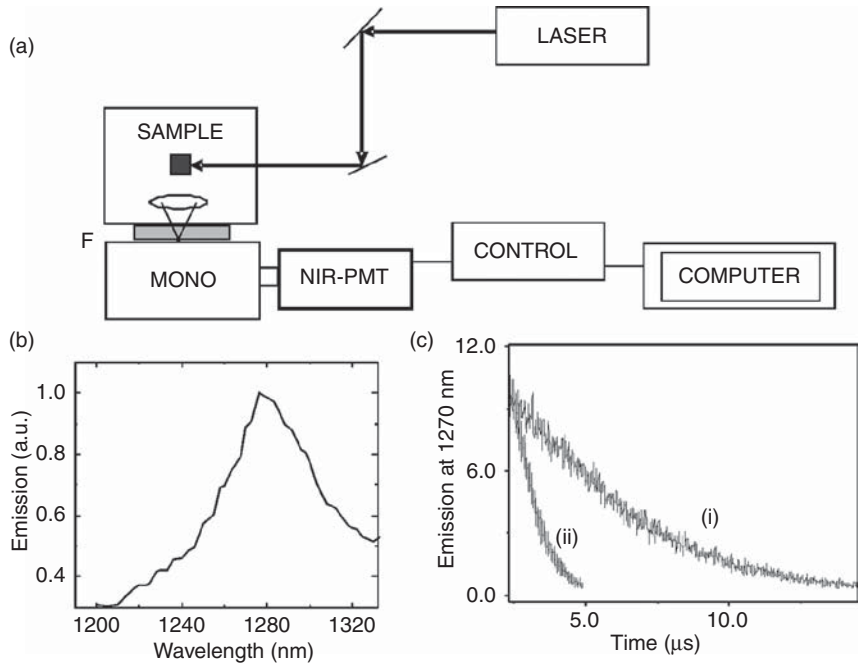


Fig. 6.3. (a) Experimental set-up to prove and study the generation and reactivity of singlet oxygen. Equipment is built with laser sources that are used to excite photosensitizer molecules that form triplet states and react with oxygen forming $^1O_2$. The light emission is filtered with silicon and/or interference filters (F), passes through a monochromator and is detected either by a NIR-PMT (faster and more sensitive) or by a Germaniun detector. (b) Characteristic NIR emission spectra of $^1O_2$ generated in aqueous solution of Methylene Blue (10 μM) after excitation at 532 nm (10 mJ/pulse, 10 Hz). (c) Transient decay of $^1O_2$ in aqueous solution, the lifetime of which is ~3 μs (i) and in aqueous solution in the presence of 1 mM sodium azide (ii) which is a commonly used agent that suppresses $^1O_2$ and consequently reduces its lifetime.

to obtain singlet oxygen by direct excitation of molecular oxygen using irradiation with an intense light source in the 0–1 transition (1070 nm), but this is a spin-forbidden process and is therefore inefficient. It requires a pressure cell in which oxygen is dissolved in a good solvent (such as hexafluorobenzene) under high pressure (140 atmospheres). It is also possible by microwave discharge in a steam of oxygen at 1–10 nm, which generates a mixture of singlet oxygen and atomic oxygen, the latter being scrubbed out by passing the gas stream over mercuric oxide (Baptista, 1998). Finally, it is possible to generate it chemically by thermal decomposition (Foote, 1968); however, the most common method for producing singlet oxygen in the laboratory is by photosensitization with a strongly absorbing dye such as methylene blue (Severino *et al.*, 2003) or chlorophyll (Krasnovskii, 1976).

Photosensitization is a process in which a molecule absorbs light and gets excited from the ground-state (PS) into a singlet, a short-lived (~10^{-9} s) excited state ($^1PS^*$) that can be deactivated by chemical reactions, or by radiative and non-radiative processes. A good photosensitizer (PS) will undergo a spin-forbidden intersystem crossing that requires a spin inversion, converting the PS to a triplet state ($^3PS^*$). The triplet states relax back to ground states via a spin-forbidden radiative pathway (phosphorescence), which imposes relatively long lifetimes. The triplet state can also be disabled by electron or proton transfer, originating radicals, as in mechanism type I (Fig. 6.4). In oxygenated environments, PS can undergo a type II photochemical process that involves energy transfer between the excited triplet state of photosensitizer ($^3PS^*$) and the triplet state of molecular oxygen ($^3O_2$), producing short-lived and highly reactive excited singlet oxygen ($^1O_2$) (Wilkinson *et al.*, 1993; Abdel-Shafi and Wilkinson, 2002; Junqueira *et al.*, 2002; Schmidt, 2006). The competition between type I and type II reactions is difficult to predict in the biological environment because the presence of biomolecules or interfaces can shift the relative rates of these processes that are observed in anisotropic solutions (Macpherson *et al.*, 1993; Baptista and Indig, 1998).

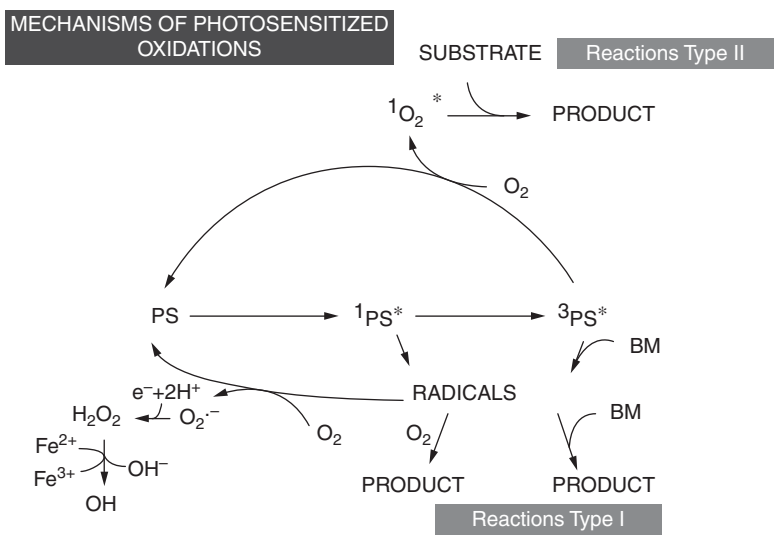


Fig. 6.4. Photosensitization mechanisms, where PS is a photosensitizer that absorbs light going to the first singlet state ($^1PS^*$), converting into a triplet state ($^3PS^*$) by intersystem crossing. The excited species, especially $^3PS^*$, can react by electron transfer forming radical species (Type I mechanism) and start radical chain reactions or react with molecular oxygen by energy transfer forming singlet oxygen (Type II mechanism). BM, biomolecules.

Plants are living organisms that survive the interaction with light. Therefore, in plant tissues, photosensitization reactions and singlet oxygen generation always compete with normal electron transfer reactions of the energy conversion process in photosynthesis. Formation of $^1O_2$ in photosystem II (PSII) of plants was invariably confirmed by the detection of its characteristic NIR emission at 1270 nm (Vass *et al.*, 1992; Telfer *et al.*, 1994). The generation mechanism involves the reduction of quinone acceptors and back-electron transfer between reduced pheophytin and oxidized P680, leading to the formation of triplet species (Durrant *et al.*, 1990). However, the details of $^1O_2$ generation are still a matter of debate and triplets derived from other photosynthetic reaction centre (RC) pigments have also been detected (Rinalducci *et al.*, 2004). The formation of $^1O_2$ in antenna-complex trimer proteins has been suggested to be the result of direct generation of $^1O_2$ by oxygen quenching of triplet chlorophyll species formed in antenna complexes after light absorption and intersystem crossing (Krieger-Liszkay, 2005; Uchoa *et al.*, 2008; Triantaphylides and Havaux, 2009). In the case of *Rhodobacter sphaeroides* RCs, Uchoa and coworkers have shown that bacteriopheophytin triplets are another possible source of $^1O_2$ (Uchoa *et al.*, 2008).

It is becoming clear that understanding and controlling singlet oxygen generation in plants may be a key factor for improving crop yield, because overproduction of $^1O_2$ can lead to photo-inhibition of photosynthesis and photo-destruction of the photosynthetic RC. Plants have developed macromolecular supra-structures and a myriad of antioxidant molecules to decrease the rate of formation of singlet oxygen by suppressing triplets and also to directly suppress singlet oxygen molecules that may be formed (Uchoa *et al.*, 2008; Triantaphylides and Havaux, 2009). The quantum yield of singlet oxygen (ϕ_Δ, number of times that singlet oxygen molecules are generated per photon absorbed), from the RCs of *R. sphaeroides* is 0.03 (Uchoa *et al.*, 2008), which is considerably smaller than ϕ_Δ calculated for PSII of plants, which was calculated to be 0.2 (Telfer *et al.*, 1994). This fact is in agreement with the smaller tendency of photo-inhibition in wild-type *R. sphaeroides* compared with plants and also with carotenoidless strains of purple bacteria (Uchoa *et al.*, 2008).

Carotenoids are especially efficient suppressors of PS triplets and of $^1O_2$. The main role of carotenoids in photosynthesis is to quench triplet states that are eventually formed in the RCs before they photosensitize $^1O_2$ formation. Carotenoids may, however, also suppress $^1O_2$ molecules that are formed in the RCs. Proof of this role for carotenoids may be obtained by comparing the efficiency of $^1O_2$ generation in different strains of purple bacteria. In RCs of *R. sphaeroides*, carotenoids are located within van der Waals distance of bacteriochlorophylls (~3.7 Å) and at 10 Å of a dimer pair of bacteriochlorophylls suppressing triplets and singlet oxygen that are formed. *Rhodopseudomonas viridis* is a strain of purple bacteria that lacks carotenoids. Consequently, one could expect a higher efficiency of singlet oxygen generation. In fact, Uchoa and coworkers have measured that the value of singlet oxygen production Φ_Δ in *R. sphaeroides* ($\Phi_\Delta = 0.03$) is half of the value of Φ_Δ in *R. viridis* ($\Phi_\Delta = 0.06$; Uchoa *et al.*, 2008).

Not only plants but also humans are affected by photoinduced $^1O_2$ generation (Lu *et al.*, 2000). Skin, hair and eyes are the most exposed areas and therefore are the tissues most prone to have photodamage (Fattorusso, 1974; Krishna *et al.*, 1991; Halliwell *et al.*, 1992; Chiarelli-Neto *et al.*, 2011). Riboflavin derivatives are widely spread in living organisms, absorb light in the UVA spectral region (Speck *et al.*, 1975; Lu *et al.*, 2000) and are known to efficiently produce $^1O_2$ ($\Phi_\Delta = 0.5$) (Wilkinson *et al.*, 1993; Morita *et al.*, 1997; Baier *et al.*, 2006). In fact, flavin co-enzymes FAD, FADH and FMN, which are of vital importance in cellular metabolism, are considered responsible for a series of endogenous photodamage in the skin, which is started by UVA absorption and generation of $^1O_2$ (Berneburg *et al.*, 1999; Kessel, 2000). It has been shown recently that melanin itself can generate $^1O_2$ under visible light exposure, showing the importance of understanding in more detail

the photosensitization processes occurring in biological surfaces in contact with sunlight (Chiarelli-Neto *et al.*, 2011).

Singlet oxygen has been shown to mediate the induction of expression of several redox defence genes (Klotz *et al.*, 2003; Luo *et al.*, 2006) as well as to cause the mitochondrial common deletion, which is associated with skin photo-ageing (Berneburg *et al.*, 1999; Wertz *et al.*, 2005). Gene expression induced by UVA in HaCaT keratinocytes is highly altered by the presence of β-carotene, an effect that was related to the suppression of $^1O_2$, as well as with direct effects of β-carotene in HaCaT cells (Wertz *et al.*, 2005). Several other workers have reported evidence of the role of singlet oxygen in UVA photoinduced damage (Krishna *et al.*, 1991; Halliwell *et al.*, 1992; Sander *et al.*, 2004). Photodamage in DNA molecules induced by endogenous and exogenous riboflavin (vitamin B2) and derivatives were reported, as well as the role of vitamin C acting as an $^1O_2$ suppressor (Cross *et al.*, 1998; Besaratinia *et al.*, 2007).

Humans have several defence systems to protect from photodamage, including a small-molecule antioxidant present in the intercellular compartments of korneocytes and melanin, which is a biopolymer that efficiently absorbs UV–VIS radiation as well as being an efficient antioxidant agent (Krol and Liebler, 1998; Callado, 2007) and also generates singlet oxygen (Chiarelli-Neto *et al.*, 2011). Even so, over-exposure to sun can lead to several skin manifestations including wrinkles, advance senescence of dermal fibroblasts and epidermal keratinocytes, which eventually can lead to the development of a cancer (Callado, 2007). Clearly these effects are more pronounced in less protected skins that have, among other differences, lower amounts of melanin (Slominski *et al.*, 2004). Overproduction of $^1O_2$ can lead to a human degenerative disease called porphyria, caused by the accumulation of porphyrins or porphyrin precursors (Straka *et al.*, 1990; Baptista, 1998). The symptoms arise mostly from effects on the nervous system and on the skin. Skin manifestations can include burning, blistering and scarring of sun-exposed areas (Bickers *et al.*, 2006).

The study of porphyria and its action mechanisms have contributed significantly to the development of therapies based on photodynamic therapy (PDT). PDT is a promising modality for the management of various tumours and non-malignant diseases, based on the combination of a photosensitizer that is selectively localized in the target tissue and illumination of the lesion with visible light, resulting in photodamage and subsequent cell death, which is mainly due to singlet oxygen (Fattorusso, 1974; Wilson *et al.*, 1992; Kalka *et al.*, 2000; Kessel, 2000; Tardivo *et al.*, 2006). The photosensitizer can also get involved in electron transfer reactions, initiating radical-induced damage in biomolecules (Baptista and Indig, 1998; Junqueira *et al.*, 2002; Severino *et al.*, 2003). Although the initial PDT protocols were very expensive and only performed in a few hospitals with expensive laser systems, recently much attention has been paid to developing inexpensive PDT protocols to treat several diseases (Tardivo *et al.*, 2006; Tardivo and Baptista, 2009).

6.4 Chemical Reactivity

Singlet oxygen involvement in photochemical reactions was first proposed by Kautsky to explain sensitized oxidation of substrates when absorbed on silica gel (Fattorusso, 1974). It is an electrophilic species, extremely reactive, that preferentially attacks highly alkylated double bonds. In biological systems, $^1O_2$ attacks membranes, proteins and nucleic acids. Its main reaction route is addition to the double bonds, although other reactions with specific targets such as sulfur compounds are also possible (Michaeli and Feitelson, 1994; Wilkinson *et al.*, 1995). Singlet oxygen attacks alkenes by three different mechanisms:

Formation of hydroperoxide (n-reaction): This mechanism involves the formation of an allylic hydroperoxide, with a concerted shift of the double bond. It is likely that the intermediate involves a

peroxide, stabilized by allylic C–H bonds (Fig. 6.5), that quickly produces the hydroperoxide (Hurst *et al.*, 1985; Poon *et al.*, 1995). The membrane phospholipids of cells and organelles contain unsaturated fatty acid derivatives that react with $^1O_2$ mainly by n-reaction (Fattorusso, 1974; Girotti, 2001; Caetano *et al.*, 2007).

Oxidative cleavage: A greater inductive electron donor in alkenes provides electron-rich alkenes with lower oxidation potential. Those alkenes generally react with $^1O_2$ to give two carbonyl fragments. In some cases, however, a mix of mechanisms occurs, forming carbonyl and hydroperoxide groups. Figure 6.6 shows examples of reactions that happen exclusively through oxidative cleavage (Fig. 6.6a) and both mechanisms (Fig. 6.6b). The mechanism of oxidative cleavage occurs by electronic reaction; the intermediate is a 1,2-dioxetane formed by 1,2-cycloaddition (Fig. 6.6c,d). These reactions are predominantly concerted [2 + 2] with both bonds being formed

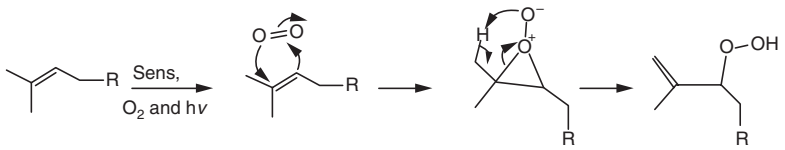


Fig. 6.5. Mechanism of n-reaction of alkenes with singlet oxygen.

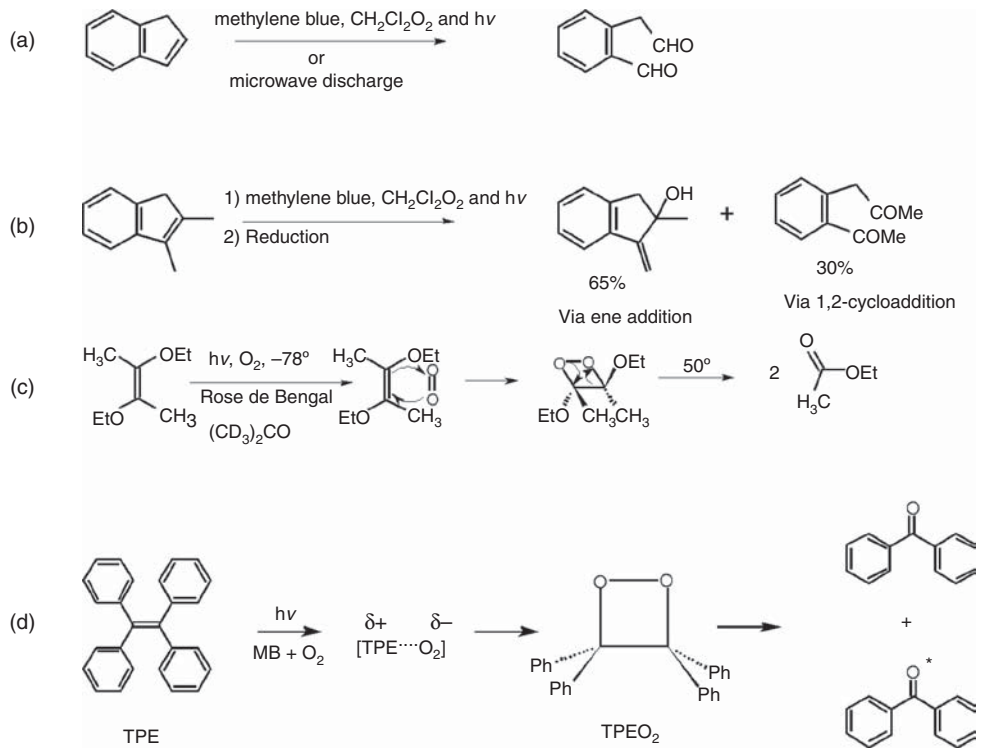


Fig. 6.6. Reaction of alkenes with singlet oxygen, oxidative cleavage (a) and by both hydroperoxide and oxidative cleavage mechanisms (b). Mechanism of oxidative cleavage showing the formation of 1,2 dioxiethane (c,d).

from the same side, suprafacial. However, an antarafacial route is also possible. The possible mechanism involves an initial charge transfer, which is conceivable where the HOMO of the electron-rich alkene is higher in energy than the lowest unoccupied molecular orbital (LUMO) of singlet oxygen. Under these circumstances an [2s + 2s] addition is allowed suprafacially, as shown in Fig. 6.7. Orbital symmetry conservation concepts are also important in understanding the thermal decomposition of 1,2-dioxethane systems, which is allowed as a concerted process, provided that one of the carbonyl fragments is formed in the excited state (Machado *et al.*, 1995).

Diels–Alder addition in conjugated dienes: The Diels–Alder reaction is a cycloaddition reaction, resulting from an electronic reorganization. Two different π bond-containing molecules react to form a cyclic compound. Each of the reactants loses a π bond and the resulting cyclic product has two new σ bonds. In a cycloaddition reaction the orbitals of one molecule must overlap with the orbitals of the other. Therefore, the frontier molecular orbitals of both reactants must be evaluated to determine the outcome of the reaction. Because the new σ bonds in the product are formed by donation of electron density from one reactant to the other, we must consider the HOMO of one of the molecules and the LUMO of the other. To perform a Diels–Alder reaction it is necessary that either the interaction of the LUMO of the dienophile (2π) and the HOMO of the diene (4π) or the HOMO of the dienophile and the LUMO of the diene occur. Singlet oxygen is a good dienophile, reacting by [4π + 2π] cycloaddition with the diene resulting in a ring system, called an endoperoxide (Di Mascio *et al.*, 1992, 1997) (Fig. 6.8).

The cyclic peroxides are often rather unstable and then decompose with explosive results. The reaction by which they are formed is a concerted symmetry allowed 4π + 2π cycloaddition. Indeed, the dienes react so rapidly that they can be used as chemical traps to divert the course of a reaction under study, suppressing $^1O_2$ and/or providing evidence for an $^1O_2$ pathway. Occasionally, the endoperoxides can be isolated and identified as intermediates. The cyclopentadiene results in a rather noxious explosive endoperoxide; however, it can be stabilized by appropriate phenyl substitution (Fig. 6.9a,b). Extended aromatic systems result in an endoperoxide that can be manipulated at room temperature (Fig. 6.9c). These reactions are reversible by thermolysis in solution, and singlet oxygen is generated (Di Mascio *et al.*, 1992, 1997).

Furans and isobenzofurans are very reactive and are sometimes used to quantify singlet oxygen generation. In both cases, the endoperoxide is an ozonide; it can be detected, isolated and characterized at low temperature (Koch and Schenck, 1966). 1,3-Diphenylisobenzofuran (DPBF) is a fluorescent molecule that possesses a highly specific reactivity towards $^1O_2$, forming an endoperoxide that decomposes to result in 1,2-dibenzoylbenzene (Fig. 6.10). Measuring the intensity decrease in absorbance or fluorescence of DPBF can follow this reaction between DPBF and $^1O_2$, which is one of the most frequent used to determine quantum yields of $^1O_2$ (Spiller *et al.*, 1998; Tada *et al.*, 2007; Rossi *et al.*, 2008).

By means of these main reactions as well as other pathways that are described in detail

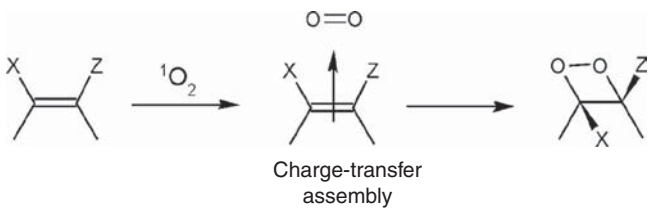


Fig. 6.7. Suprafacial attack of singlet oxygen with charge-transfer assembly.

elsewhere (Foote, 1976; Kessel, 2000; Girotti, 2001; Schweitzer and Schmidt, 2003), $^1O_2$ can react with biomolecules and cause damage to their structures and functions. Each main group of bio-structures, i.e. membranes, proteins and nucleic acids, shows distinct patterns of molecular damage and decrease of bio-functionality. Membrane damage is initiated by attack to the double bond of unsaturated lipids through the formation of lipid hydroperoxides (Girotti, 2001); proteins have some susceptible amino acids that are usually initially oxidized, i.e. tryptophan, cysteine, methionine, tyrosine and histidine as well as some redox prosthetic groups (Foote, 1976). DNA is damaged particularly in guanosine residues forming 8-oxoguanosine, which facilitates the continuation of the oxidation progress because 8-oxoguanosine is much more susceptible to oxidation than guanosine itself (Devasagayam *et al.*, 1991). In this chapter most emphasis is given to membrane damage.

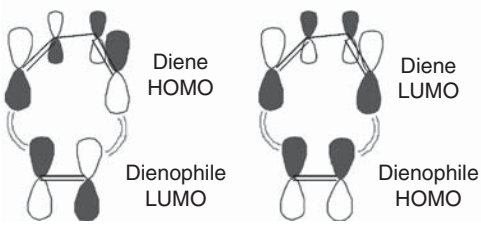


Fig. 6.8. Frontier molecular orbital analysis of a [4 + 2] cycloaddition reaction.

Lipid hydroperoxides (LOOH) are the initial species generated when membranes made of unsaturated lipids are attacked by singlet oxygen (Fig. 6.11). The progression of the peroxidation reactions depends on the formation of peroxyl (LOO•) and alkoxyl (LO•) radicals. Metals are able to catalyse the conversion from LOOH to LOO• and LO•. Also PSs that engage in type I reactions or any PS in the presence of LOOH can also catalyse the formation of LOO• and LO• (Gantchev *et al.*, 2003). The progression of peroxidation reactions can cause severe damage to membrane properties and quickly lead the cell to necrotic death. Indeed, the efficiency of cell death caused by singlet oxygen seems to be related to the efficiency with which the PS interacts with membranes (Pavani *et al.*, 2009).

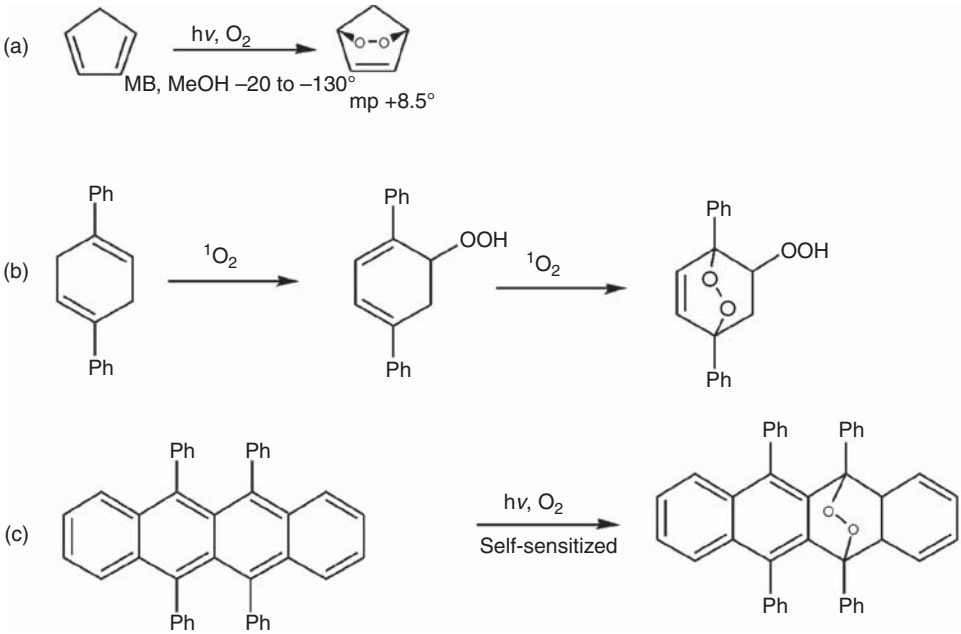


Fig. 6.9. Cycloadditions using singlet oxygen as dienophile.

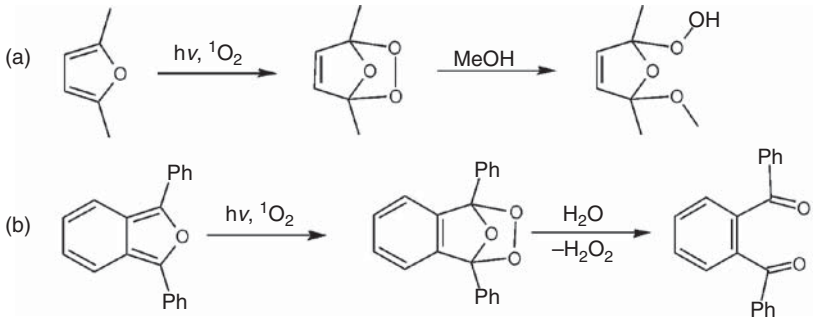


Fig. 6.10. Mechanism of reaction of 2,5-dimethylfuran (a) and DPBF (b) with singlet oxygen.

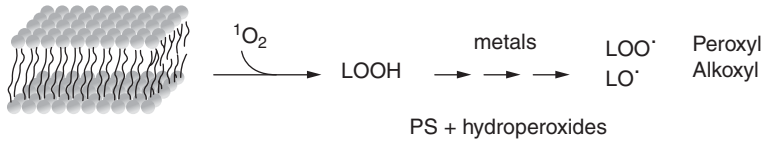


Fig. 6.11. Main routes of membrane damage through lipid peroxidation.

Fatty acids quench $^1O_2$ with rate constants from 10^4 to 10^5 $M^{-1}s^{-1}$ (Krasnovsky *et al.*, 1983). The values of the rate constants depend on how electron-rich the double bound is and the solvent capacity to stabilize the reaction intermediaries that control the reaction velocity (Machado *et al.*, 1995; Girotti, 2001). Membrane destruction can be microscopically observed by experiments with giant vesicles (GUVs) (Riske *et al.*, 2009). In the manuscript by Caetano and coworkers, GUVs were destroyed after a few minutes of exposure to $^1O_2$ (Caetano *et al.*, 2007). The mechanism of membrane damage was attributed to lipid chain break with formation of short-chain amphiphiles.

Although this general picture is well accepted, it is important to understand the effect that the progressive increase in lipid hydroperoxide concentration has on the membrane properties. Lipid hydroperoxides have a more hydrophilic character than the lipid itself because of the hydroperoxide group incorporated into the acyl chain. The peroxidized chain tends to migrate to the bilayer surface (Riske *et al.*, 2009). This change causes an increase in area per lipid, disturbing chain packing order and increasing membrane fluctuations. Riske *et al.* (2009) observed that peroxidation of as much as 60% of the lipids was still compatible with intact membranes. Using the rate of singlet oxygen production of the photosensitive molecule, it was estimated that the efficiency of the oxidative process is 0.0037. This work suggested a possible protection role of the lipid structure in keeping the membrane integrity even at high levels of molecular damage.

6.5 Deactivation of Singlet Oxygen: Kinetics and Mechanisms

Singlet oxygen can be suppressed (quenched) by two main mechanisms: physical (there is no formed product) and chemical (there is an oxidized product), whose constants are represented as k_q and k_r, respectively (Wilkinson *et al.*, 1995). The total or observable quenching constant (k_Q) is the sum of three terms: k_d, which is the pseudo-first-order rate constant for solvent deactivation of $^1O_2$ and $k_q[S]$ and $k_r[S]$ that account for the physical and chemical quenching, respectively, of substrate S over $^1O_2$.

Several molecular interactions can lead to the physical deactivation of $^1O_2$: energy

transfer in which the excitation energy can be transferred directly into high energy vibrational modes that are quickly transferred to the surroundings (e.g. the OH effect) (Foote and Denny, 1971); energy transfer to another excited state that would then be funnelled to surrounding energy vibrational modes (e.g. the carotenoids) (Schmidt, 2004), and reversible electron transfer reactions (Schweitzer and Schmidt, 2003) (Fig. 6.12). These processes explain why $^1O_2$ has short lifetimes in water and protic solvents (methanol and ethanol; Table 6.1) and also why the azide anion is an excellent quencher of singlet oxygen (Wilkinson *et al.*, 1995). The OH group is present in a large number of singlet oxygen suppressors and has one of the highest vibrational energy levels; its overtone transition is near to the lower energy level of $^1O_2$ (96 kJ/mol) (Krasnovskii, 1976), facilitating the electronic-vibrational coupling. Foote and Ogilby showed in the 1970s that $^1O_2$ has longer lifetimes in deuterated solvents and performs photo-oxidation reactions, unravelling the possible role of $^1O_2$ (Foote and Denny, 1971; Ogilby and Foote, 1982).

The other two physical processes through which $^1O_2$ decays without forming products are the reversible electron and energy transfer transition states ($\neq$) formed with the azide ion (Catalan *et al.*, 2004) and β-carotene (Wilkinson *et al.*, 1995; Schmidt, 2004), respectively (Fig. 6.12). Both mechanisms lead $^1O_2$ to decay to the ground state ($^3O_2$). The constants involved in these processes are diffusion controlled (~10^{10} mol.l^{-1}.s^{-1}). In others words, there are effective and fast ways to suppress $^1O_2$. It is important to emphasize that under physical quenching the excited state energy of singlet oxygen is dissipated in the surroundings as heat. Both oxygen and the quencher agents return to their original state.

Chemical quenching includes all the reactions described in the last section, plus the simple electron transfer reactions that convert singlet oxygen into anion radical superoxide. Molecules that present adequate E^0 values and that stabilize well positive charges are good candidates to suppress singlet oxygen by this specific mechanism (Oliveira *et al.*, 2011), which depends on solvent stabilization of the involved intermediates (Machado *et al.*, 1995; Schweitzer and Schmidt, 2003). In general, the interaction of singlet oxygen with molecules favours more than one mechanism simultaneously. For example, carotenoids are the most efficient known singlet oxygen suppressors reacting mostly by physical mechanisms, although chemical quenching is also observed. Proteins, enzymes and DNA also quench singlet oxygen by chemical and physical mechanisms (Lu *et al.*, 2000; Schmidt, 2004).

Nature looks for strategies to protect itself from these oxidation reactions. For protection against sunlight, humans have melanin to avoid light from reaching the

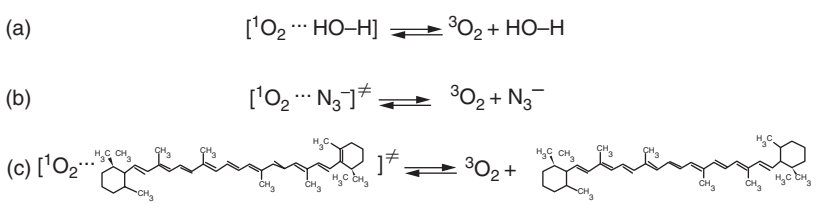


Fig. 6.12. Main deactivation routes of singlet oxygen: (a) electronic-vibrational coupling, (b) reversible electron transfer and (c) energy transfer transition states.

Table 6.1. Lifetimes of singlet oxygen in various solvents ($^1\Delta_g$).

Solvent	H_2O	MeOH	C_6H_6	CS_2	CCl_4	C_6F_6	D_2O	Air, 1 atm.
τ(μs)	3	7	24	200	700	3,900	70	~76,000

photosensitizer pigments. Plants, on the other hand, make use of their secondary metabolites in an appropriate and necessary way to suppress $^1O_2$ and other oxidizer agents that could lead to destruction of biological systems.

Plants metabolize countless antioxidants, such as flavonoids, anthocyanins, ascorbic acid, carotenoids and vitamin A. The antioxidants can be found in every vital structure of plants, and they are part of their defence mechanisms. Many of these substances such as lycopene and those made by the shikimic acid pathway, are not biosynthesized in animals, but have important roles in the life of humans. The challenge now would be to understand how these chemical structures that have been known to exert different roles in human health could be playing their role by deactivating singlet oxygen.

It is important to mention that singlet oxygen is also used to synthesize natural products. One example of the action of $^1O_2$ in synthesis of natural products is the synthesis of ascaridol (Fig. 6.13a), which is a component of chenopodium oil (Schenck, 1954). Schenck and Ziegler, who carried out this synthesis in 1941, expressed the view that ascaridol may be generated in the plant by a photosensitized reaction involving chlorophyll, rather than by an enzymatic process. Another example is in germacranolides: costumonolide undergoes photooxygenation to obtain peroxycostumonolide using methylene blue as photosensitizer (Fig. 6.13b) (El-Feraly, 1977).

6.6 Carotenoids

Carotenoids are molecules constituted of polyene conjugated systems and are shown to be involved in the prevention of several human diseases, such as cancer, coronary heart disease, age-related eye retina macular degeneration and eye lens cataracts (Foote and Denny, 1968; Di Mascio *et al.*, 1989; Conn *et al.*, 1991; Trekli *et al.*, 2003). The mechanism of disease prevention seems to be primarily related to the prevention of peroxidation processes. The ability of several carotenoids to quench singlet oxygen has been studied, and their general antioxidant activities have also been extensively studied (Gust *et al.*, 1993; Edge *et al.*, 1997; Frank, 2003). As mentioned above, the quenching of $^1O_2$ by carotenoids is mainly due to an efficient energy transfer through transition states process (triplet–triplet

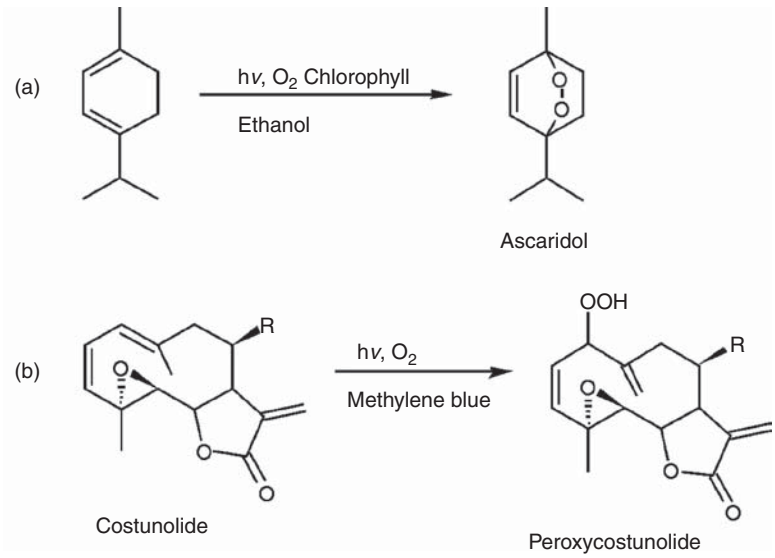


Fig. 6.13. Two examples of natural oxidative processes via singlet oxygen in plants.

coupling process) (Schmidt, 2004). The triplet energy of β-carotene (88 kJ/mol) is just slightly smaller than that of $^1O_2$ (96 kJ/mol), which allows it to suppress $^1O_2$ (Foote and Denny, 1968; Ogilby and Foote, 1982).

Over 600 different carotenoids have been identified up to now. Several synthetic pathways lead to different molecules with conjugated polyene systems. Phytoene is synthesized from the union of two C_{20} geranylgeranyl pyrophosphatate units and from that lycopene is obtained by enzymatic hydrogen elimination (Fig. 6.14). β-Carotene, the cyclic derivative of lycopene, is a precursor of vitamin A and is an orange substance found in carrots, mangoes, apricots and sweet potatoes. β-Carotene OH derivatives (such as lutein; Fig. 6.14) are responsible for the yellow colour in the leaves in the autumn (Biesalski, 2007). β-Carotene is used in the food industry and in the industry of food supplements. Bixin results from lycopene oxidation and it is the main carotenoid found in the seed coat of urucum fruits (*Bixa orellana L.*; Fig. 6.15), which is cultivated in tropical countries of South and Central America, Africa and Asia.

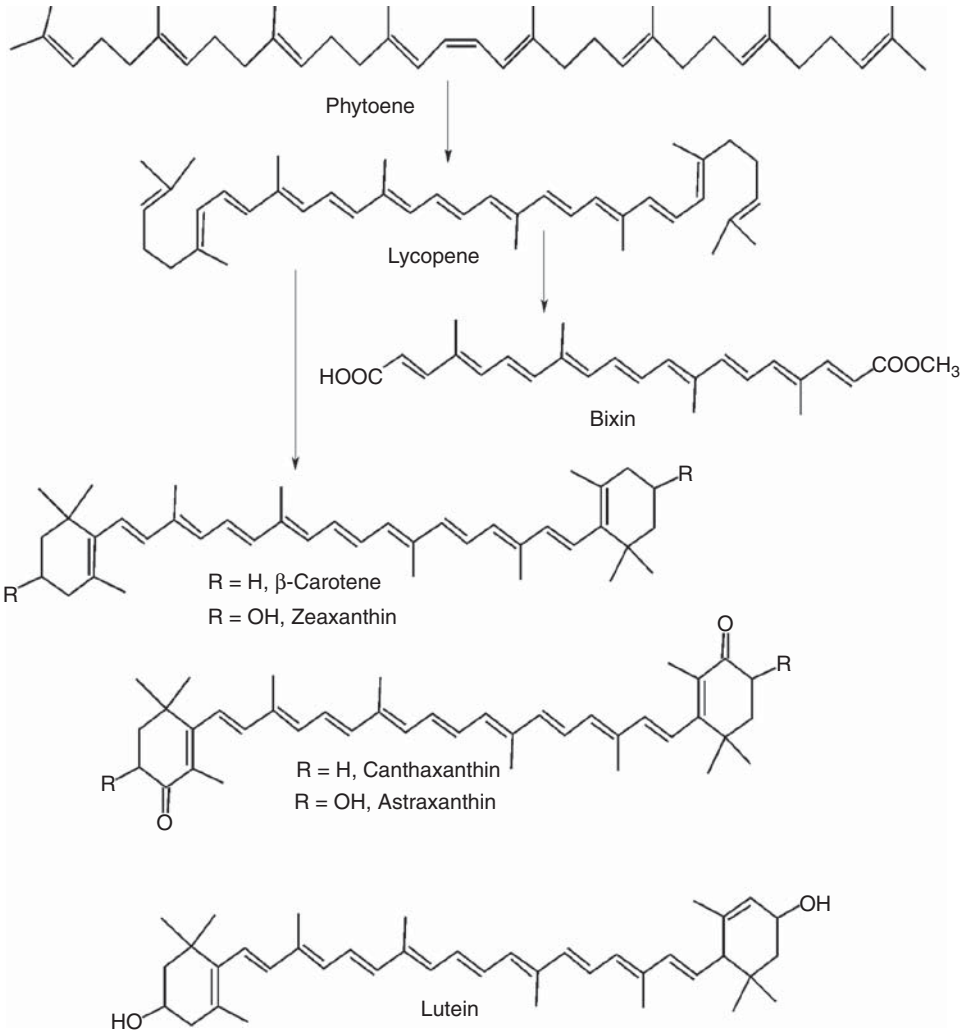


Fig. 6.14. Chemical structures of phytoene, lycopene, β-carotene and some other dietary carotenoids.

Fig. 6.15. Urucum fruits (*Bixa orellana* L.).

Urucum extract has large amounts of carotenoids with a large diversity of chemical structures (other structures are bixin, zeaxanthin, canthaxanthin, astaxanthin and lutein; Fig. 6.14). It has been used by South American native civilizations as a colouring dye for the human body and food, guaranteeing increased photo-protection from the caustic effects of photosensitized reactions in the tropical and equatorial areas. Nowadays, carotenoids are used in food supplements and have been shown to protect against sunlight-induced erythema in human skin (Gollnick *et al.*, 1996; Biesalski and Obermueller-Jevic, 2001) and photo-induced immunosuppression (Fuller *et al.*, 1992; Herraiz *et al.*, 1998).

Because photosensitized oxidations may be mediated both by singlet oxygen and by free radicals, multiple antioxidant actions of carotenoids may contribute to its protective effect (Krinsky, 1989). Both singlet oxygen and free radicals produced during photosensitized oxidations can cause cellular damage by reacting with DNA and proteins, or by inducing lipid peroxidation. Carotene seems to protect against photosensitized tissue injury both by scavenging free radicals and by quenching singlet oxygen (Krinsky, 1979). In fact, β-carotene is clinically used to prevent photosensitized tissue damage in humans with porphyria (Mathews-Roth, 1986).

The analysis of specific products has been used to monitor singlet oxygen quenching in experimental models of phototoxicity, and has demonstrated that carotenoids can quench singlet oxygen by physical and chemical processes. The photo-oxidation of 0.1 M of 2-methyl-2-pentene is inhibited by 95% by 10^{-4} mol.l^{-1} of β-carotene (Foote *et al.*, 1970). The rate constant for quenching of $^1O_2$ by β-carotene is $k_q = 1 \times 10^{10}$ l. mol^{-1}s^{-1} that accounts for most of its suppression ability (Schmidt, 2004). The reaction products of singlet oxygen oxidation of β-carotene shows a series of compounds including ionone and apocarotenal, endoperoxide, dihydroperoxides, chain-cleavage and carbonyl-containing products (Fig. 6.16). The formation of the endoperoxide (β-carotene-5,8-endoperoxide) is typical of 1,4-cycloaddition reactions of singlet oxygen with *cis*-dienes. This confirms in an unequivocal way that the oxidation mechanism is through $^1O_2$ (Stratton *et al.*, 1993).

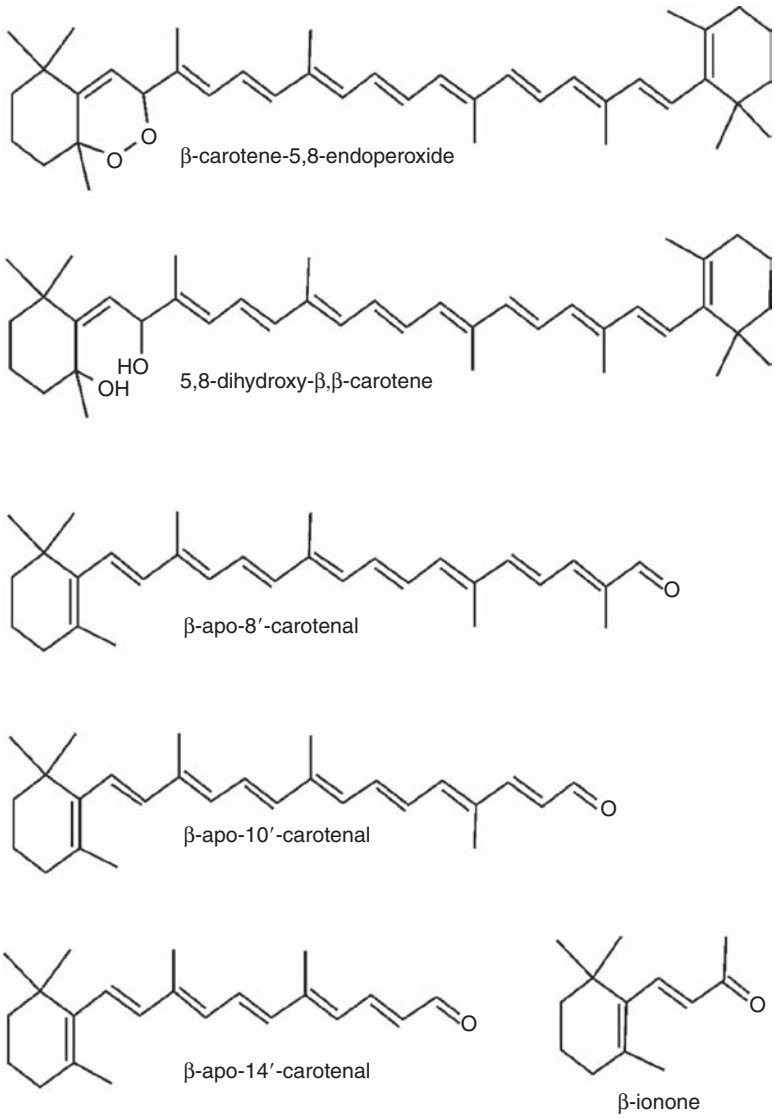
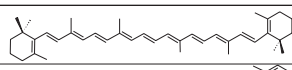
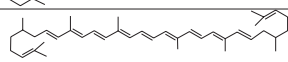
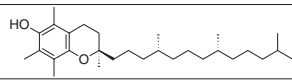
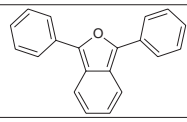
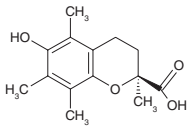
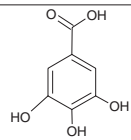
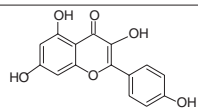
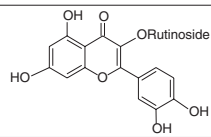
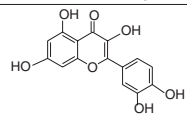
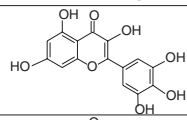
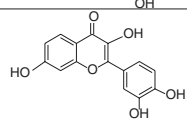
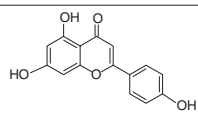


Fig. 6.16. Structures of some of the $^1O_2$ oxidation products of β-carotenoids.

6.7 Flavonoids as Singlet Oxygen Suppressors

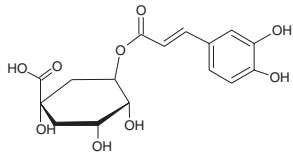
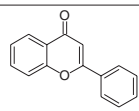
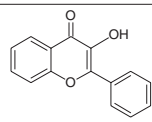
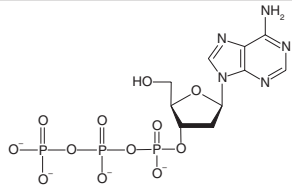
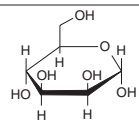
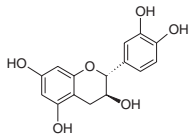
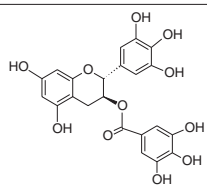
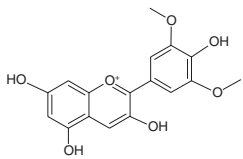
Flavonoids are another family of naturally occurring substances that should be considered in terms of antioxidant and singlet oxygen suppression activities. They are important secondary metabolites that protect plants against bacteria and fungi as well as from UV–VIS photo-induced oxidation reactions, and continue to be the main target of research in plant bio-prospection (Veitch and Grayer, 2008; Pedriali *et al.*, 2010). In the past decade, scientists started to become aware of the fact that $^1O_2$ suppression is an important aspect of flavonoid activity. The values of $^1O_2$ rate constants (K_Q), physicochemical properties, and molecular structure of flavonoid, carotenoid and catechin molecules, Trolox (a vitamin-E derivative), ATP, glucose, histidine, ascorbic acid and diphenylbenzofuran (DPBF), are presented in Table 6.2.

Table 6.2. Singlet oxygen total quenching rate constants (K_Q), HOMO energies and LogP for a series of flavonoids and other antioxidants.

Antioxidant		E_{HOMO} (eV)	10^{-8} K_Q ($mol.l^{-1}.s^{-1}$)	LogP Experimental (theoretical)
β-Carotene			100	(15.5)
Lycopene			200	(15.5)
α-Tocopherol			2.0	(11.9)
L-Histidine			0.9	(–1.3)
DPBF			200	(6.5)
Trolox		–8.90	1.2	2.4 (3.0)
Gallic acid		–9.32	0.05	(0.9)
Ascorbic acid		11.20	2.0	(–2.4)
Kaempferol		–9.04	0.005	2.72 (2.1)
Rutin		–9.06	1.2	–2.7 (1.8)
Quercetin		–9.05	4.6	2.26 (2.1)
Myricetin		–9.06	5.1	(–0.5)
Fisetin		–8.99	0.01	2.20 (2.1)
Apigenin		–9.16	0.28	2.62 (2.1)

Continued

Table 6.2. Continued.

Antioxidant		E_{HOMO} (eV)	10^{-8} K_Q ($mol.l^{-1}.s^{-1}$)	LogP Experimental (theoretical)
Chlorogenic acid		–9.23	0.022	(–0.4)
Flavone		–9.29	<0.003	
Flavonol		–8.97	0.053	(1.5)
NaN_3			3.0	
ATP			0.0004	
Glucose			0.0001	
Melanin			1.0	
Tannic acid			0.22	
Catechin			0.11	
EGCG			1.5	
Malvidin			5.6	

EGCG, epigallocatechin gallate.

By using these data, it is possible to draw important conclusions about structure–activity relationships and the mechanisms of $^1O_2$ (Mukai *et al.*, 2005; Nagai *et al.*, 2005; Yamaguchi *et al.*, 2005). A quick glance at Table 6.2 shows that carotenoids are clearly the most efficient singlet oxygen suppressors, the efficiency of which is similar to that of DPBF, which is an efficient singlet oxygen probe. All other molecules have non-bonding electron pairs like those found in the azide ion, polyphenols, DNA bases and proteins.

Polyphenols are also good $^1O_2$ suppressors. By comparing the value of k_Q of myricetin ($k_Q = 5.1 \times 10^8$ mol.l^{-1}.s^{-1}) with that of flavone ($k_Q < 0.003 \times 10^8$ mol.l^{-1}.s^{-1}) it is clear that the number of available OH groups is an important factor, suggesting that physical quenching is taking place (Mukai *et al.*, 2005; Nagai *et al.*, 2005). It seems to be necessary for these molecules to have aryllic O–R groups that carry non-bonding electron pairs that favour complexation with singlet oxygen by forming a charge-transfer complex. It therefore seems that the suppression mechanism of flavonoids is basically due to the process of the reversible electron transfer reaction; however, before reaching that conclusion, one should analyse further the data shown in Table 6.2. We have thus presented data as two figures, in which k_Q is plotted as a function of the number of O–R groups (Fig. 6.17) and HOMO energies (Fig. 6.18).

Note that there is a clear relationship between the number of O–R available groups and the value of k_Q (Fig. 6.17). However, it is not only the number of O–R groups that matters because sugars have lots of OH groups, but are poor singlet oxygen suppressors (Table 6.2). In fact, one can notice that the energy of the HOMO orbitals is also important (Fig. 6.18), in agreement with the mechanism of the electron transfer reaction. Therefore, a higher HOMO energy allows the formation of a charge transfer complex and reversible electron transfer reaction. Tannins are an exception to this rule, once they have a large number of O–R groups, and we could expect more efficient singlet oxygen suppression than is observed. We suspect that this low value of k_Q of tannins is

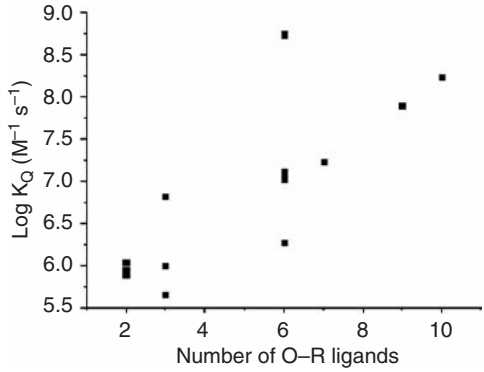


Fig. 6.17. Number of oxygens with a non-bonding electron pair in linear relationship with Log K_Q. (Adapted from Mukai *et al.*, 2005 and Nagai *et al.*, 2005.) O–R are chemical groups in which O is bound to an H or alkyl group.

due to the formation of aggregates and low availability of the O–H groups that could deactivate singlet oxygen. However, it is important to mention that, besides their low $^1O_2$ suppression constant measured *in vitro*, tannins have shown expressive protection against singlet oxygen induced damage in DNA, indicating that other factors besides k_Q should be considered in understanding protection against specific oxidative damages.

Another factor that should be considered in terms of the efficiency of singlet oxygen suppression is the partition in the aqueous and organic phases. Rutin and myricetin are good suppressors of singlet oxygen, but present a logP lower than zero (Table 6.2). It means that they should work well in solution but in compartmentalized systems and membranes their protection efficiency should be small. On the other hand, quercetin is an efficient singlet oxygen suppressor and has a logP value of 2.26, indicating that it will partition well in membranes and therefore have a better potential to protect them from oxidative damage.

Although physical quenching is clearly the most efficient mechanism of interaction between $^1O_2$ and flavonoids, chemical products have also been detected indicating that chemical quenching also takes place. $^1O_2$ cannot react by Diels–Alder with benzofuran; however, it can attack the 2–3 double

bond of the hydroxyflavanone quercetin (Fig. 6.19a) to afford a depside. This type of reaction may proceed through a hydroperoxide intermediate, which cyclizes and decomposes with the loss of carbon monoxide or carbon dioxide.

The oxidation of quercetin to the corresponding depside also occurs in biological systems. However, the precursors of flavonoids, chalcones, conjugated with carbonylic groups favour the Diels–Alder reaction with this species of oxygen (Fig. 6.19b). A somewhat related reaction is the oxidation of chalcones, which are the biogenetic precursor of aurones. Sensitized photooxygenation of a chalcone proceeds through dioxetane to yield aurone.

Vitamine E (α-tocopherol and similar compounds) is also a relatively efficient singlet oxygen suppressor (Table 6.2) and is widely used as an antioxidant agent (Huang *et al.*, 2005; Molyneux, 2007; Nenadis *et al.*, 2007). Trolox, which is a water-soluble derivative of vitamin E, is also used

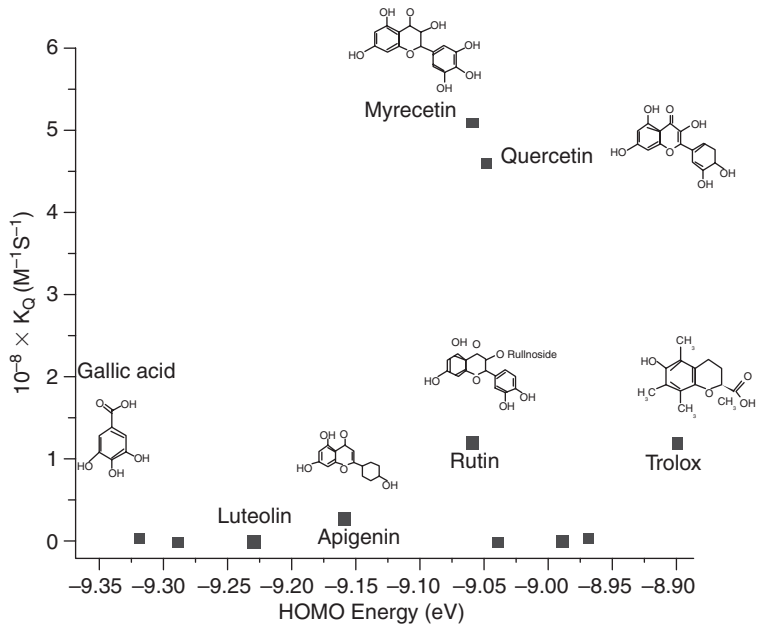


Fig. 6.18. K_Q versus HOMO energy for a series of flavones, flavonols and similar structures.

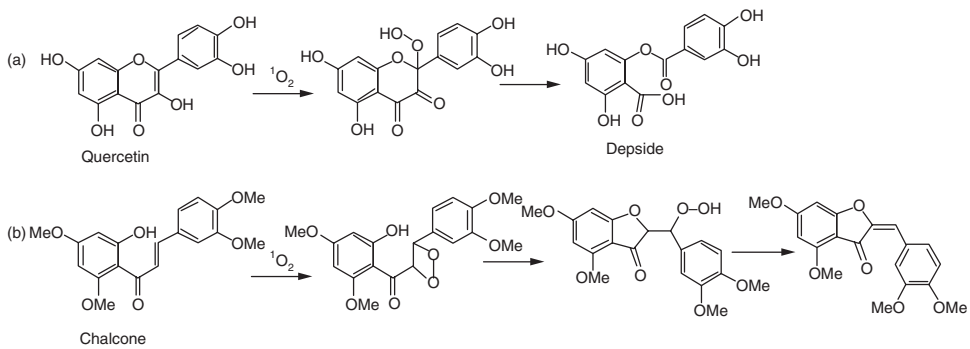


Fig. 6.19. Mechanism of attack of $^1O_2$ in the flavonoids (a) quercetin and (b) chalcon.

as an antioxidant and efficiently quenches singlet oxygen. The chemical structure of these molecules as well as the range of their k_Q values ($1–2 \times 10^8$ $mol.l^{-1}.s^{-1}$) suggest that the mechanism of singlet oxygen suppression of this class of molecules is similar to that observed for flavonoids, i.e. due to a reversible electron transfer reaction. Both α-tocopherol and β-carotene are hydrophobic molecules with a high tendency to localize in membranes. β-Carotene is, however, a much more efficient $^1O_2$ suppressor, suggesting that it should be more efficient in protecting membranes from damage initiated by $^1O_2$. In fact, Stratton and Liebler have shown that, in concentration conditions similar to those found physiologically, β-carotene was highly effective in protecting against formation of oxidation products of membranes and α-tocopherol was ineffective (Stratton and Liebler, 1997).

Several other groups of molecules are also known to suppress $^1O_2$ with high efficiency so that they could be considered as antioxidant owing to their $^1O_2$ suppression abilities. Examples can include ascorbic acid, histidine and catechins (Table 6.2). Betanidines, found in high concentration in beetroot, also seem to hold promising properties to protect against damage resulting from $^1O_2$ (Bonacin *et al.*, 2009).

Carotenoids, which suppress $^1O_2$ through triplet–triplet energy transfer, present k_Q values that are around two orders of magnitude larger than those observed for the other groups of molecules cited in Table 6.2. However, it does not mean that one should disregard the $^1O_2$ suppressor abilities of flavonoid derivatives and catechins, because these molecules may be present in different concentrations and they certainly have different cellular and extracellular localization domains. In fact, aqueous extracts of plants, namely *Andrographis paniculata* and *Swertia chirata*, significantly protect against oxidative damage induced by various oxidants including $^1O_2$ (Tripathi *et al.*, 2007). The difference in polarities among these groups of molecules suggests possible synergistic roles of carotenoids and flavonoids. Carotenoids are extremely lipophilic (logP>15) and should either work inside the structure of a membrane protein or inside the membrane itself, whereas most of the flavonoids are hydrophilic and should work in aqueous interfaces or in aqueous solutions.

6.8 Conclusions

Singlet oxygen plays important roles in photo-induced damage in animals and plants, causing damage to human skin and decreasing crop yields. $^1O_2$ is particularly generated by the absorption of UVA–VIS photons by naturally occurring photosensitizers, whose triplets react with molecular oxygen. The reactivity of $^1O_2$ with electron-rich double bonds allows it to react with several biomolecules, changing their chemical structure and altering their functions. In terms of the effects in membranes, formation of lipid hydroperoxide is the first step in lipid peroxidation that can progress to chain break and loss of membrane integrity. Nature has developed a series of chemicals that protect biomolecules from the damage caused by $^1O_2$. The main quenchers found in nature suppress $^1O_2$ by physical mechanisms. The fact that all these molecules are well known antioxidant agents and that they have high efficiency in quenching $^1O_2$ indicates that an important part of their antioxidant activity is due to the suppression of $^1O_2$. Carotenoids are the most efficient $^1O_2$ suppressors and the mechanism of suppression is by triplet–triplet energy transfer. Their main site of localization is hydrophobic environments, contrary to flavonoids and catechins, which are also efficient $^1O_2$ suppressors, whose main quenching mechanism is due to reversible electron transfer reactions. The different environments and suppressor mechanisms of these molecules suggest a possible synergistic action of carotenoids, flavonoids and catechins in suppressing $^1O_2$ and helping cells to keep homeostasis under conditions of redox misbalance.

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7 Antioxidant Activity and Chemical Composition of Colombian Propolis

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7.1 Introduction

Propolis, also known as bee glue, is a resin-like product collected and elaborated by bees (*Apis mellifera*) from parts of plants, buds and exudates; it is transformed and used by bees to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders. The word propolis is derived from the Greek pro-, for or in defence, and polis-, city, i.e. defence of the city (or the hive) (Ghisalberti, 1979). In recent years there has been a renewed interest in the composition of propolis, a substance that can be regarded as a potential natural source in folk medicine and in the chemical industry, particularly because the chemical diversity of propolis makes it a valuable source of new biologically active compounds.

Propolis has been employed extensively since ancient times; for example, Egyptians benefited from the anti-putrefactive properties of propolis in order to embalm their dead. Also, it was used as an antiseptic and cicatrizant agent by the Greek and Roman physicians, and Incas employed propolis as an anti-pyretic agent; in addition, the London pharmacopoeias of the 17th century listed propolis as an official drug (Sforcin and Bankova, 2011). Today, numerous studies have proven its versatile pharmacological activities, and it is traditionally employed in natural medicine, apitherapy, biocosmetics and nutraceuticals with many purposes (Bankova *et al.*, 2000). Most of the studies carried out have proved that this material has many properties such as antibacterial (Marcucci *et al.*, 2001), antifungal (Yang *et al.,* 2011), antiviral (Kujumgiev *et al.*, 1999), anti-inflammatory (Hu *et al.*, 2005), anti-ulcer (Primon de Barros *et al.,* 2008), hepatoprotective (Shimizu *et al.,* 2004) and anti-tumoural (El-khawaga *et al.,* 2003), among many others. There is also much evidence of the antioxidant property of propolis, which is mainly attributed to phenolic compounds, especially flavonoids (Russo *et al.*, 2004; Gregoris and Stevanato, 2010; Cottica *et al.,* 2011).

Although the biological activity of bee glue, and especially its activity against microorganisms, is always present in samples from different geographic and climatic

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zones, this activity was the result of a different chemical composition (Kujumgiev *et al.*, 1999). It is well known that biological activities of propolis are strongly related to their chemical composition, and this is probably the major problem when attempting to use propolis as an ingredient in phytotherapy, since the chemical composition depends on the surrounding flora, harvest time, the collection technique used and the bee species that produce them (Pereira *et al.*, 2002).

The chemical composition and biological activities of propolis have been studied extensively in Europe and some countries of America, especially in Brazil and Argentina. As might be expected, the conclusion is evident: there are appreciable differences in propolis' qualitative and quantitative composition, and this is strongly related to their particular biological and pharmacological properties (Bankova, 2009). Despite this, there are limited studies on the chemical composition and evaluation of biological activities of Colombian propolis and this is one of the reasons why we are interested in the physical, chemical and biological characterization of propolis from different regions of Colombia that have not been studied previously.

7.2 Physicochemical Characterization of Propolis from Colombia

The precise composition of raw propolis varies with the source. It possesses a pleasant aromatic smell and varies in colour, depending on its source and age. Raw propolis is generally composed of 50% resins, 30% wax, 10% essential oils and aromatic compounds, 5% pollen and 5% other substances (Burdock, 1998). The proportion of these types of substances varies and depends on the place and time of collection. Moreover, it has been established that this chemical variability depends on different factors, such as: (i) exudates and bud from plants where bees collect it; (ii) substances contained in bee secretions; and (iii) materials introduced during propolis' elaboration process (Marcucci, 1995). Chemical studies of this bee product have identified at least 300 compounds (Castro, 2001), including flavonoids, aromatic acids and their esters, aldehydes and ketones, fatty acids and their esters, terpenes, steroids, amino acids, polysaccharides, hydrocarbon compounds, alcohols and many others present in traces (Bankova *et al.*, 2000).

Due to the different uses and properties reported for propolis, the medical, pharmaceutical, food and cosmetics industries have been focused on this resinous substance. As a consequence, it has caused the necessity to create quality control and standardization protocols for raw propolis and propolis extracts. Many countries, such as Brazil (Ministerio, 1999) and Argentina (Norma IRAM-INTA, 2004), have developed different methodologies to evaluate quality parameters such as minimum soluble ethanol resin content (EEP) (30%), maximum content of wax (40%) and insoluble residues (25%), among others. Additionally, phenolic compounds (5%) and flavonoids (0.5%), which are commonly considered to be responsible for biological activities, are also analysed.

In our studies with propolis we have evaluated some physicochemical parameters for samples collected in the North (Atlantic Department) and Andean (Antioquia Department, South-west and Bajo Cauca subregions) regions of Colombia. The results of these analyses are shown in Table 7.1. Our results are compared with international standard values reported in Brazilian and Argentinian regulations.

In the results obtained there are considerable variations in humidity, ashes, EEP, waxes and insoluble material content. For most of the samples tested, the wax content was the only parameter that is in compliance with Brazilian and Argentinian regulation values (less than 5%) (Ministerio, 1999; Norma IRAM-INTA, 2004). Although raw propolis samples showed low EEP values, these extracts could present other different types of bioactive principles in their composition with different biological properties that can confer them a quality status.

Table 7.1. Physicochemical parameters for propolis samples from Atlántico and Antioquia Departments.

Propolis sample	EEP (%)[a]	Wax content (%)[a]	Insoluble material (%)[a]	Humidity (%)[a]	Ash content (%)[a]
Atlántico[b]	8.48–24.21	1.77–6.07	55.57–77.93	5.74–11.69	0.35–3.86
Bajo Cauca[b]	6.46–25.84	2.00–33.60	36.53–89.82	1.35–8.18	0.91–2.33
South-west[c]	10.44–40.64	45.11–76.42	8.28–28.23	1.82–4.96	0.16–4.77
Brazilian regulation	Min. 35.0	Max. 25.0	Max. 40.0	Max. 8.0	Max. 5.0
Argentinian regulation	Min. 30.0	Max. 40.0	Max. 25.0	Max. 10.0	Max. 5.0

[a]Minimum and maximum values found; [b]analysis carried out according to Brazilian regulation protocols; [c]analysis carried out according to Argentinian regulation protocols.

7.3 Propolis: Botanical Origin and Secondary Metabolites

As we previously mentioned, propolis is a bee product made from different tissues and plant exudates. The chemical composition of this material is strongly influenced by their geographical origin and the botanical sources used to produce it, which can vary according to range of temperature and latitude. In temperate zone countries of the Northern hemisphere, propolis samples have a relatively constant qualitative composition because bees mainly use for their elaboration buds from poplars (*Populus nigra*, Salicaceae and *Populus nigra,* Pyramidalis) (Ghisalberti, 1979; Greenaway *et al.*, 1990) and only a few plant sources such as birch and elm. Similarly, in eastern Australia bees employ endemic species of the genus *Xanthorrhoea* (Bankova *et al.*, 2000).

In tropical regions, where there is no poplar existence, bees can find alternative sources for resin production (Bankova *et al.*, 2000). In countries from Central and South America honeybees employ plant resins from *Clusia* species to produce red propolis. Resins from *Clusia minor* and *Clusia rosea* are employed by Venezuelan and Cuban bees, respectively. In Brazil botanical sources of resin that originates green propolis are *Araucaria heterophylla, Clusia major, Clusia minor, Araucaria angustifolia, Eucalyptus citriodora* and especially *Baccharis* species (Greenaway *et al.*, 1990). Now it is clear, and widely proved, the appreciable differences in chemical composition between propolis from tropical regions and temperate regions. Furthermore, owing to the richness and variety of tropical flora, the propolis' chemical composition is highly complex and variable. In Table 7.2 the chemical characteristics of propolis from different regions of the world are presented.

7.3.1 Chemical profile of propolis from South America

For the complete characterization of propolis, different techniques such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) have been used. In routine analysis, ultraviolet absorption (UV) with a diode array detector (DAD) is commonly employed, but the use of hyphenated techniques, mainly gas chromatography-mass spectrometry (GC-MS), is growing.

As an example, in the analysis of propolis samples Ahn *et al.* (2007) using HPLC-DAD and MS identified caffeic acid (**1**), *p*-coumaric acid (**2**), ferulic acid (**3**), 3,4-dimethoxycinnamic acid (**4**), pinobanksin 5-methyl ether (**5**), pinobanksin (**6**), pinocembrin (**7**), pinobanksin 3-acetate (**8**), caffeic acid phenetyl ester (**9**), cinammyldeneacetic acid (**10**), cinnamyl caffeate (**11**), chrysin (**12**), galangin (**13**) and tectochrysin (**14**) (Fig. 7.1). Some of these compounds have also been found in propolis from Europe (Banskota *et al.,* 2002; Uzel *et al.,* 2005), Argentina and Uruguay (Kumazawa *et al.*, 2002).

Table 7.2. Chemical composition of propolis according to their geographic origin.

Geographic origin	Major constituents	References
North America, Europe, New Zealand, non-tropic regions of Asia	Poplar propolis: flavones, flavanones, cinnamic acids and their esters	Nagy *et al.* (1986), Greenaway *et al.* (1988), Markham *et al.* (1996), Bankova *et al.* (2000)
Russia	Birch propolis: flavones and flavonols (not the same as in poplar type)	Popravko (1978)
Mediterranean countries: Sicily, Greece, Crete, Malta	Mediterranean propolis: diterpenes mainly labdane-type acids	Trusheva *et al.* (2003), Melliou and Chinou (2004), Popova *et al.* (2010)
Okinawa, Taiwan, Indonesia	Pacific propolis: C-prenyl-flavanones	Chen *et al.* (2008), Kumazawa *et al.* (2008), Trusheva *et al.* (2011)
Brazil	Green propolis: prenylated p-coumaric acids, diterpenic acids Other propolis: acetophenone, lignans, triterpenoids and anacardic acid derivatives	Bankova *et al.* (2000), Salatino *et al.* (2005), Albuquerque *et al.* (2007), Silva *et al.* (2008)
Cuba, Brazil and Mexico	Red propolis: isoflavonoids (isoflavans, pterocarpans)	Daugsch *et al.* (2008), Lotti *et al.* (2010)
Cuba, Venezuela	Polyprenylated benzophenones	Cuesta-Rubio *et al.* (2002), Trusheva *et al.* (2004), Márquez *et al.* (2005)
Cuba	Yellow propolis: triterpenoids (lanosterol, α- and β-amyrin, lupeol, cicloartenol) and flavonoids	Márquez *et al.* (2010)
Chile	Lignans	Valcic *et al.* (1998)
Peru	Triterpenoids (lupeol acetate and β-amyrin acetate)	Bracho *et al.* (2009)
Argentina	Polyphenols (quercetin, kaempferol, pinocembrin, chlorogenic acid, chalcone), lignans and epoxylignan	Chaillou and Nazareno (2009), Agüero *et al.* (2010, 2011)
Uruguay	Flavonoids (pinobanksin 3-(2-methyl) butyrate), carboxylic acids, and phenolic acid esters(2-methyl-2-butenyl ferulate)	Kumazawa *et al.* (2002)

Led by Brazil, in the tropical regions of South America, propolis production has rapidly increased. In countries with a great biodiversity, however, such as Venezuela, Ecuador and Colombia, chemical and biological studies on propolis are scarce. Propolis types from tropical zones are mainly composed of prenylated p-coumaric acids, acetophenones, lignans, diterpenes and triterpenes (Bankova, 2000).

From Brazilian propolis, Banskota *et al.* (1998) isolated and characterized prenylated compounds (3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid (**15**)), labdane type diterpenes (agathic acid (**16**), agathalic acid (**17**), cupressic acid (**18**)), benzofurans (tremetone (**19**)) and flavonoids (betuletol (**20**)), among others (Fig. 7.2).

Furthermore, Banskota *et al.* (2000) identified from Brazilian propolis two benzofurans, named benzofuran A (**21**) and B (**22**) (Fig. 7.3), which showed moderate cytotoxicity against carcinogenic cells.

Marcucci *et al.* (2001) analysed a propolis sample from Paraná, Brazil, and reported four phenolic compounds named as 3-prenyl-4-hydroxycinnamic acid (**23**), 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran (**24**), 3,5-diprenyl-4-hydroxycinnamic acid (**25**) and 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopiran (**26**) (Fig. 7.4).

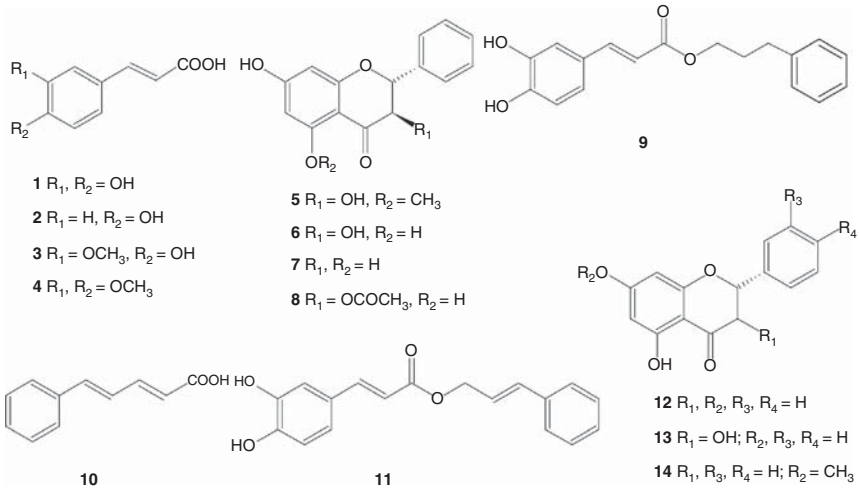

Fig. 7.1. Main compounds from propolis samples identified by HPLC using PDA and MS detectors. For names please refer to the text.

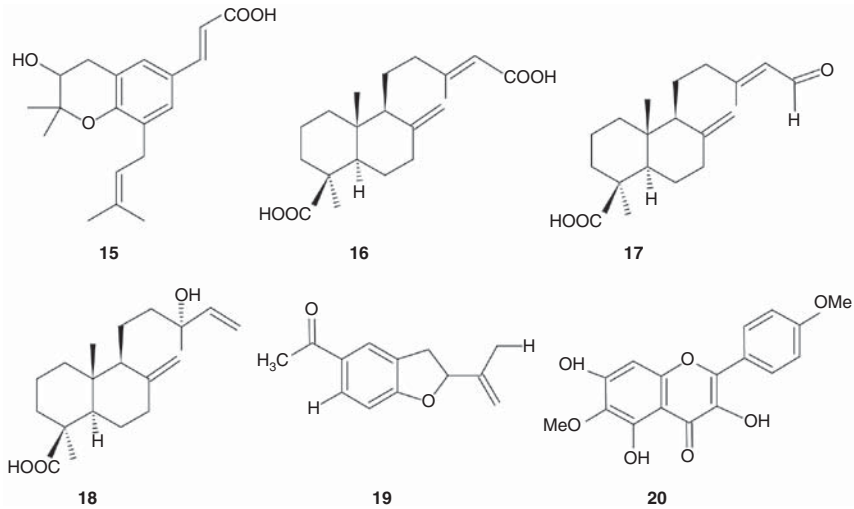

Fig. 7.2. Compounds isolated from Brazilian propolis (Banskota *et al.,* 1998). For names please refer to the text.

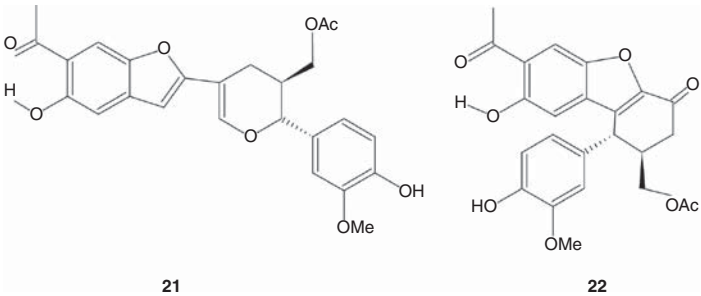

Fig. 7.3. Benzofurans isolated from Brazilian propolis (Banskota *et al.,* 2000). For names please refer to the text.

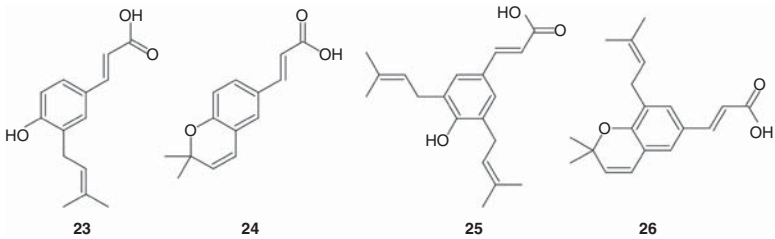

Fig. 7.4. Phenolic compounds isolated from Brazilian propolis (Marcucci *et al.,* 2001). For names please refer to the text.

Although most of the Brazilian studies of propolis were carried out with green propolis, Trusheva *et al.* (2006) studied a red propolis sample collected in the Northern region of Brazil. Phenylpropanoid derivatives (*trans*-anethol, methyl eugenol, *trans*-methyl isoeugenol, elemicin, *trans*-isoelemicin), triterpenic alcohols (α-amyrin, β-amyrin, cycloartenol, lupeol), isoflavonoid, pterocarpans, naphtoquinones and a mixture of prenylated benzophenones were reported for this sample.

Even though there are not many studies on the chemical composition of Venezuelan propolis, some researchers have described the presence of prenylated benzophenones. In 2004 Trusheva *et al.* isolated two prenylated benzophenones named 18-ethyloxy-17-hidroxy-17,18-dihydroscrobiculatone A (**27**) and B (**28**) (Fig. 7.5), and also found scrobiculatones A (**29**) and B (**30**), which were previously reported by Porto *et al.* (2000) from flower resin of *Clusia* species. These compounds showed antibacterial activity and moderate toxicity against *Artemia salina*.

Because of the geographical isolation of Chile, the botanical sources of Chilean propolis are exudates of endemic plants belonging to the families Asteraceae, Anacardiaceae, Rosaceae, Rhamnaceae, Monimiaceae and Lauraceae. Valcic *et al.* (1998) showed that the main compounds present in Chilean propolis were lignans (**31**–**35**) (Fig. 7.6). Additionally, flavonoids have been reported (Astudillo *et al.*, 2000).

In the past ten years Argentinian propolis collected from several locations have been studied. In general terms, polyphenols (flavanones, flavones, flavonols and chalcones) and organic acids (coumaric acid, ferulic acid and cinnamic acid) were the major constituents found (Agüero *et al.*, 2010). These results suggested that the botanical origin of propolis samples were poplar trees. Moreover, in samples of propolis from the Andean region of Argentina, lignans and epoxylignans were identified and the botanical source was associated with the medicinal plant *Larrea nitida* Cav. (Zygophyllaceae) (Agüero *et al.*, 2011).

7.3.2 Chemical profile of propolis from Colombia

In the Colombian context, although products based on this beehive product are available on the market, there is scarce information about the chemistry of propolis. At the moment only a few studies based on the chemical composition of Colombian propolis have been done. For these reasons we are interested in characterizing, chemically and biologically, propolis collected in Colombia. Some of the studies that we have carried out are summarized below.

From dichloromethane and petroleum ether/metanol extracts of a propolis sample of the Apiary of the National University of Colombia, (LIMA), located in the city of Medellín, Antioquia, Colombia, were purified three labdane type diterpenes that were identified by ^{1}H and ^{13}C mono- and bi-dimensional NMR techniques as isocupresic acid (**36**), agathadiol (**37**) and epi-13-torulosol (**38**) (Fig. 7.7) (Meneses *et al.*, 2009).

Additionally, Palomino *et al.* (2010) established, using GC-MS, a chemical

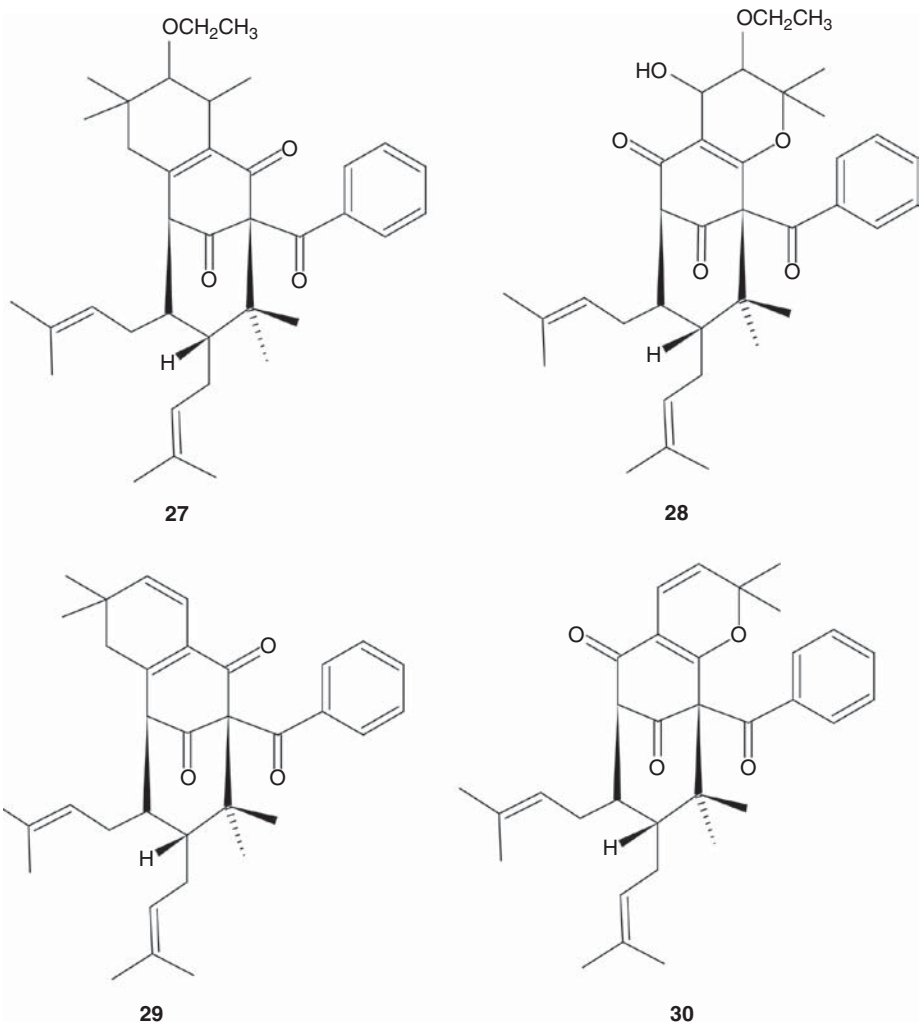

Fig. 7.5. Prenylated benzophenones isolated from Venezuelan propolis (Trusheva *et al.*, 2004). For names please refer to the text.

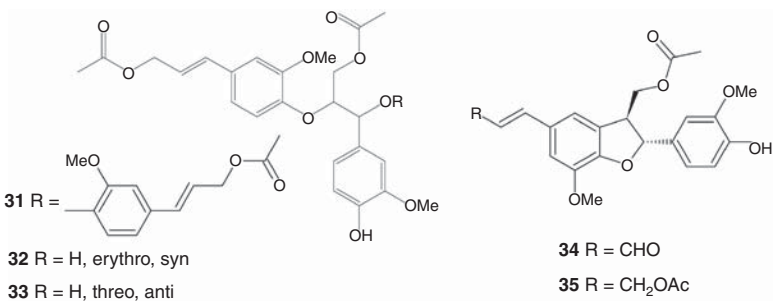

Fig. 7.6. Lignans isolated from Chilean propolis (Valcic *et al.,* 1998).

profile of an ethanolic propolis extract collected from La Unión municipality, located in the South-east region of Antioquia (Fig. 7.8).

From this analysis the following compounds were detected: (i) methyl and ethyl esters of saturated and unsaturated fatty acids (peaks 10, 17, 18); (ii) various sesquiterpenes previously reported in European propolis (cadinene, muurolene, cadinol and muurolol) (Silici and Kutluca, 2005; Melliou *et al.*, 2007); (iii) six pentacyclic triterpenes (peaks 6, 9, 20, 24, 29) such as cicloartenol acetate and 7-friedouren-3-ol, which showed a characteristic fragmentation pattern with peaks at m/z 177 (84%), 191, 341, 205 (Budzikiewicz *et al.*, 1963), and 3-lupanol acetate which showed an ion at m/z 191; (iv) two types of diterpenes (bicyclic type labdane) and a tricyclic acid (abietic acid). Similarly, a later study established the chromatographic profile of some propolis samples collected in Antioquia's Department yielding the results shown in Table 7.3.

These results showed evident differences in the chemical composition of propolis from the Antioquia Department and reflect the complexity of the mixture. We can appreciate that these samples are mainly

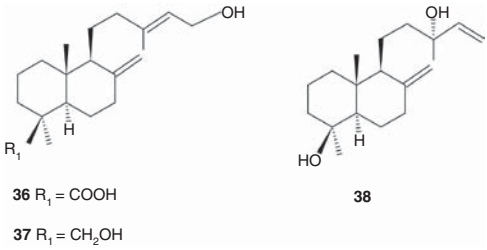

Fig. 7.7. Labdane type diterpenes isolated from Antioquia Department propolis.

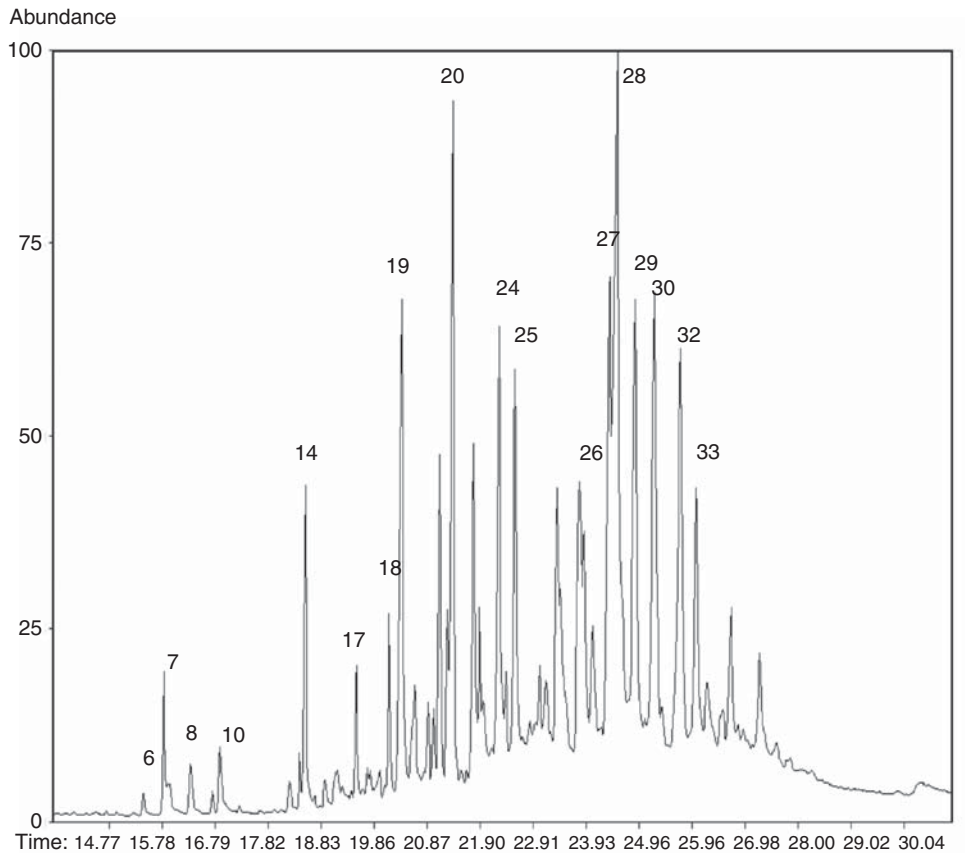

Fig. 7.8. An enlarged version of a gas chromatographic profile of a propolis sample collected in La Unión municipality.

Table 7.3. List of the compounds identified by GC-MS from ethanolic extracts of Antioquia's municipalities propolis samples.

		Relative abundance (%)				
Compounds*	Rt (min)	B-R	C-T	C-R	LU-T	UN-T
Fatty acid						
Palmitic acid	39.7	7.7	0.5	1.5	1.5	0.8
Oleic acid	47.6	14.4	–	1.2	0.2	1.1
Retinoic acid	65.8	–	–	1.8	–	–
Stearic acid	47.9	2.7	–	–	–	–
Oleic acid ethyl ester	47.7	0.5	–	–	–	–
Palmitic acid ethyl ester	38.7	0.3	–	–	–	–
Sugars and polyols						
D-Fructose	26.7	4.0	–	–	–	–
Glucopyranose	31.6	1.8	–	–	–	–
D-Galactose	34.7	–	–	2.6	–	–
Erythritol	17.9	2.2	5.4	32.0	8.5	0.1
Xylitol	26.6	–	0.6	3.7	0.4	4.4
Arabinofuranose	29.1	–	2.2	2.4	0.6	–
Manose	34.3	–	0.6	–	1.2	0.7
D-Altrose	26.9	–	–	13.6	–	–
Inositol	27.6	–	–	1.9	–	–
Glucitol	30.7	–	–	1.8	–	–
Manitol	34.3	–	0.9	–	–	–
Galactitol	31.6	–	–	–	–	1.2
Alcohols						
Glycerol	63.6	3.9	–	–	–	–
Butanol	73.2	–	5.2	–	–	–
Acids						
Butanedioic acid	10.9	2.0	–	0.3	0.1	0.4
Propanoic acid	68.7	–	1.5	–	–	–
Malic acid	16.8	–	–	1.1	–	–
Hydroxymalonic acid	17.7	–	–	0.1	–	–
Pentenoic acid	63.4	–	–	–	1.1	–
Terpenes and steroids						
Dehydroabietic acid	58.8	1.7	–	0.8	–	0.9
Abietic acid	50.3	–	0.9	–	5.3	1.2
Lupan-3-ol benzoate	47.9	–	–	–	3.5	–
Lupan-3-ol acetate	52.2	–	–	–	0.8	–
Betuline	59.8	–	–	–	6.1	–
β-amirin	89.2	9.2	–	–	–	–
Labdane type diterpene (not identified)	49.4	–	5.0	0.6	–	2.6
Manool	44.3	–	–	–	–	5.5
Methyl cis-comunate	54.5	–	–	–	–	0.6
Sclarene	57.9	–	–	–	13.7	–
Isopimaric acid	64.7	–	13.7	4.7	1.9	–
Ursane-20(30)-ene-3,16-diol	46.7	–	0.6	–	–	–
Gurjunene	25.6	–	–	–	–	1.0
Pimaric acid	60.1	–	26.8	–	–	–
17α-Methyl-17β-hidroxy-1,4,6-androstatrien-3-one	54.2	–	–	–	0.8	–

Continued

Table 7.3. Continued.

Compounds*	Rt (min)	Relative abundance (%)				
		B-R	C-T	C-R	LU-T	UN-T
7-Hydroxy-4-androstene-3,17-dione	54.7	–	–	–	3.5	–
Steroid (not identified)	61.0	–	–	–	–	30.6
Ergosterol	61.4	–	–	–	5.4	–
3-Hydroxy androstan-17-one	65.1	–	–	–	–	1.4
Pimaral	62.6	–	–	–	–	2.9
Cycloartane-type triterpene	68.3	33.4		–	–	–
Methyl abietate	63.7	–	–	–	–	19.1
17-Hydroxy-4-androstene-3-one	56.6	–	–	–	2.5	–
Flavonoids						
4′,5-Dihidroxy-6,7-dimethoxy-flavone	48.7	1.3	–	–	–	–
5-Hidroxy-3′,4′,6,7-tetramethoxy flavone	51.0	–	1.4	–	–	–
Phenols						
p-(Hydroxymethyl) phenol	20.2	–	–	0.1	–	–
p-Hydroxyphenyl acetic acid	24.3	–	–	0.7	–	–
Melibiosa (Disacárido)	65.8	–	–	–	1.4	–
4-Hydroxyphenyl ethanol	19.2	–	–	–	0.3	–
Others						
Unidentified compound	62.9	–	–	–	19.5	–
Vismiaquinone	48.9	–	–	–	–	1.7

B-R, Betania-scraping; C-T, Caldas-trap; C-R, Caldas-scraping; LU-T, La Unión-trap; UN-T, Apiary Universidad Nacional-Medellín.

composed of fatty acids and their ester derivatives, pentacyclic triterpenes and diterpene acids. The methodology employed did not allow the detection of flavonoid and phenolic type compounds.

In conclusion, the Colombian propolis is somewhat comparable to Brazilian propolis originating from *Araucaria* species, which is also rich in labdane diterpene acids (communic, isocupressic, acetyl-isocupressic, imbricatoloic) (Bankova *et al.*, 1996). In the same way, the chemical profile is similar to the profile of yellow Cuban propolis, which is rich in triterpenes and sterols (Cuesta-Rubio *et al.*, 2007; Márquez *et al.*, 2010). These studies on the chemical composition of Colombian propolis could help to establish criteria for the classification and the quality control of national propolis samples.

7.4 Total Phenolic Content, Total Flavonoid Content and Antioxidant Activity of Propolis

In biological systems, cells are commonly faced with free radicals and non-free-radical species, which are produced by essential endogenous processes (detoxification, chemical signals, energy supply, etc.) or by external sources (Valko *et al.*, 2007; Rodrigo, 2009). These chemical substances, commonly referred to as reactive oxygen species (ROS) and reactive nitrogen

species (RNS), are extremely reactive, and when there is an over-production of them a damaging process known as 'oxidative stress' occurs (Wang *et al.*, 2011). There is a large body of evidence that shows a strong relationship between ROS and pathological and physiological disorders such as cancer, neurodegenerative disorders, hypertension, diabetes, hyperlipidemia, cardiovascular diseases, inflammation, autoimmune disorders and so on (Fu *et al.*, 2011). The reason for this is the considerable number of harmful effects generated by ROS species in the organism's biomolecules, cells, tissues and organs (Dryden *et al.*, 2005; Dimitrios, 2006). To overcome these problems there is an incessant search for novel antioxidants, and it is commonly accepted that substances with antioxidant activity can contribute to preventing diseases, promoting health and serve as a template for the development of potentially novel therapeutic agents (Venkat-Ratnam *et al.*, 2006).

Additionally, at present most of the antioxidant's arsenal available in pharmaceutical, food and cosmetic industries has been substantially reduced, mainly owing to three factors: (i) toxicity problems associated with synthetic molecules used as antioxidants; (ii) a strong tendency of consumers to buy products based on natural ingredients; and (iii) an increased move by manufacturers from synthetic to natural origin antioxidants to comply with people's demands (Pokorny, 2001).

In the search for novel antioxidant molecules from nature, many compounds have been tested and have shown potential as antioxidant agents (Ng *et al.*, 2000; Wootton-Beard and Ryan, 2011). The most common natural compounds associated with antioxidant capacity are polyphenols that include hydroxycinnamic acids, flavonoids, hydroxybenzoic acids, dihydrochalcones, isoflavonoids and tannins. Furthermore, some other types of metabolites have also demonstrated good activity (quinines, betalains, organosulfides, steroids, saponins, etc.) (Uttara *et al.*, 2009; Aiyegoro and Okoh, 2010). These natural compounds are highly appreciated because their traditional uses as food and medicinal products has allowed them to be considered safer and healthier than synthetic products (Shi *et al.*, 2001; Krishnaiah *et al.*, 2011), but some problems related to low bioavailability and delivery formulation have arisen (Venkat-Ratnam *et al.*, 2006). That is why more studies to find new, secure, healthy and cheap antioxidant compounds must be carried out.

In the Colombian context, although products based on this resinous material (propolis) are available on the market, there is scarce information about their chemistry and their biological activities. At the moment, both physicochemical characterization and evaluation of biological activities are considered two good parameters for establishing propolis' quality. Moreover, it is well known that depending on the geographic origin of propolis samples, the biological activities are different (Popova *et al.*, 2011). For these reasons, we are interested in chemical and biological characterization of propolis collected in some regions from Colombia.

One of the most analysed activities for propolis, together with antimicrobial activity, is antioxidant capacity. In many countries several studies have been carried out to analyse this particular activity (Kumazawa *et al.*, 2004; Laskar *et al.*, 2010). These reports have shown a good correlation between polyphenolic content and antioxidant activity (Russo *et al.*, 2004; Choi *et al.*, 2006; Ahn *et al.*, 2007), and that is the reason why this class of compound is usually associated with this activity. Owing to the number of factors that can affect the antioxidant capacity (species and reaction systems), and the diverse number of mechanisms through which antioxidant substances exert their action, numerous methods to test the antioxidant properties of biological origin samples are currently available (Badarinath *et al.*, 2010; Cíz *et al.*, 2010). The methods that we used to evaluate antioxidant activity on propolis were (to see more detailed information about each method please refer to Huang *et al.* (2005)):

1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), a stable radical that is used to evaluate the

scavenging properties of antioxidant compounds. In a methanol solution, DPPH acquires a purple colour that changes to yellow when an antioxidant substance is present.

2. Trolox Equivalent Antioxidant Capacity Assay (TEAC), a technique similar to DPPH assay. In the presence of sodium or potassium persulfate, an ABTS (2,2´-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)) stable radical cation is generated. This oxidant species ($ABTS^{\bullet-}$) is blue, but in the presence of an antioxidant compound turns to the $ABTS^{2-}$ colourless form.

3. Ferric ion reducing antioxidant power assay (FRAP), a colorimetric assay carried out in acidic conditions to evaluate the total antioxidant capacity of compounds. This technique uses a $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ complex, (TPTZ = 2,4,6-tri(2-pyridil)-s-triazine), that, in the presence of an antioxidant agent, is reduced to its ferrous form $[Fe(II)\text{-}(TPTZ)_2]^{2+}$, yielding a blue colour.

4. Oxygen radical absorbance capacity (ORAC), an antioxidant scavenging activity assay against peroxyl radicals formed by heating AAPH (2,2´-azobis(2-amidino-propane) dihydrochloride), a free-radical generator compound. In the method, radicals formed attack fluorescein (a fluorescent probe). Therefore, if a high fluorescence value remains, it is assumed that the antioxidant is acting.

Furthermore, spectrophotometric assays are the reference methods for determination of total phenolic content and total flavonoid content, because they are fast and cheap. Popova *et al.* (2004) validated some procedures for the quantification of three of the main bioactive groups of metabolites identified in propolis (phenols, flavones and flavonols, flavanones and dihydroflavonols). The authors assert that the joint quantification of compounds with similar structures correlates much better with biological activities and provide better information than quantification of individual compounds.

7.4.1 Total phenolic content

Phenolic compounds or polyphenols are widely recognized for showing a great number of biological activities, such as anticancer, anti-inflammatory, immunomodulatory, analgesic and antioxidant, among others (Gómez *et al.*, 2006). Propolis are rich in these kind of substances; however, the phenolic concentration from one sample of propolis to another can vary considerably depending on the origin. Therefore, the presence of some biological activities can be present or absent.

The reasons mentioned above support the determination of total phenolic content (TPC) as a quality-control parameter for propolis. At present, this determination is done by using the Folin–Ciocalteau colorimetric method. This methodology is based on the formation of a coloured substance that absorbs at 760 nm, which is produced by the oxidation of the phenolic compounds with phosphoric molibdotungstate (Singleton and Rossi, 1965). Some countries, such as Brazil and Argentina, have established official quality control standards for raw propolis. They have a minimum limit of 50 mg AE/g of Ethanol Extract of Propolis (EEP) for phenolics (Ministerio, 1999). Furthermore, many studies have reported a direct correlation between TPC and antioxidant and antimicrobial properties of propolis (Kumazawa *et al.*, 2004; Choi *et al.*, 2006; Moreira *et al.*, 2008).

7.4.2 Total flavonoid content (TFC)

Flavonoids are the main group of phenolic compounds. Because of the many biological and therapeutic activities attributed to them, these compounds have been the object of many scientific studies. Although other phenolic compounds are also involved, high flavonoid content is thought to account for much of the activities in propolis.

An aluminium chloride ($AlCl_3$) colorimetric assay is a method commonly used to evaluate total flavonoid content (TFC) in propolis samples (Woisky and Salatino, 1998; Ahn *et al.*, 2004, 2007; Kumazawa *et al.*, 2004; Marquele *et al.*, 2005; Choi *et al.*, 2006; Gómez *et al.*, 2006; Soleo de Funari *et al.*, 2007; Moreira *et al.*, 2008). In this

method the formation of complexes between $AlCl_3$ with keto and hydroxyl groups is evaluated at a wavelength of 425 nm (Kumazawa *et al.*, 2004). On the other hand, the quantification of flavanones and dihydroflavanones is based on the interaction of these compounds with 2,4-dinitrophenylhydrazine (DNFH) in acidic medium and the consequent formation of coloured phenylhydrazones (Popova *et al.*, 2004).

7.4.3 Specific absorbance of UV spectrum

The $E^{1\%}_{1cm}$ absorption value is one of the physicochemical parameters used to evaluate the quality of propolis, because, as was previously mentioned, most of the activities of this beehive product are attributed to the presence of phenolic compounds such as flavonoids and caffeic acids (Mohammadzadeh *et al.*, 2007). These classes of compounds show a wavelength of maximum absorption of between 240 and 350 nm.

7.4.4 Total phenolic content and total flavonoid content of propolis from Colombia

Because there are not many studies on the chemical characterization and antioxidant activity of propolis from Colombia, we have performed some analyses in order to evaluate these parameters. We collected samples from municipalities of two departments of Colombia: Atlántico, located in the Caribbean region, and Antioquia, in the Andean region (Fig. 7.9). The results that we have achieved were grouped by department and are described in the following sections.

Fig. 7.9. Regions of Colombia where propolis samples were collected.

Propolis from the Antioquia Department

Antioquia is one of the 32 departments in Colombia. It is located in the North-west region of Colombia, crossed by two of the main mountain chains, Cordillera Central and Cordillera Occidental. This provides many varied geographical zones broadly classified as mountainous and non-mountainous. Antioquia is divided into nine regions that comprise 125 municipalities.

SOUTH REGION. All the propolis samples studied in this section come from municipalities belonging to different South sub-regions of the department. Betania (B) belongs to the Southern east region, La Unión (LU) is placed in the East region, and Caldas (C) and Apiary Universidad Nacional-Medellín (UN) are in the Aburra's Valley region. For each sample, the method of collection is indicated by R (scraping) or T (plastic trap).

In the analysis of $E^{1\%}_{1cm}$ values of propolis the values range from 72 to 390. The highest $E^{1\%}_{1cm}$ value was shown by propolis from the Betania municipality (B-R, 390) and the lowest for UN-T (72). As an overview of the results obtained, TPC and TFC values of the samples analysed vary widely, ranging from 22.11 ± 0.54 to 75.22 ± 1.35 mg gallic acid (GAE)/g of EEP and 4.75 ± 0.01 to 42.37 ± 0.18 mg quercetin (QE)/g of EEP, respectively (see Table 7.4).

Some countries, such as Brazil and Argentina, have established official quality control standards for raw propolis. They have a minimum limit of 50 mg GAE/g of EEP and 5 mg QE/g of EEP for phenolics and flavonoids, respectively (Ministerio, 1999; Norma IRAM-INTA, 2004). According to both regulations, propolis from B-R, LU-T and C-T are in accordance with international minimum requirements.

Propolis from UN-T had the lowest values in phenolic and flavonoid content (Table 7.4). These low values could be due to the presence of labdane-type diterpenes as predominant compounds in this sample (Meneses *et al.*, 2009). Propolis from B-R showed the highest phenolic and flavonoid content values. These were higher than Colombian propolis samples previously analysed but lower than propolis samples from Europe, North America and Asia (Kumazawa *et al.*, 2004).

NORTH REGION. Bajo Cauca is a sub-region located in the North-east area of Antioquia's Department. It is composed of six municipalities: Caucasia, El Bagre, Nechí, Tarazá, Cáceres and Zaragoza. Propolis samples

Table 7.4. Total phenolic content, total flavonoid content and antioxidant activity of EEP from different municipalities of South region of Antioquia Department, Colombia.

Sample	Specific absorbance of UV spectrum $E^{1\%}_{1cm}$ (λ)	Total phenolic content[a]	Total flavonoid content[b]	DPPH TEAC[c] ± SD (μmol/g)	ABTS TEAC[c] ± SD (μmol/g)	FRAP AEAC[d] ± SD (μmol/g)
Caldas C-T	162 (296)	67.11 ± 0.89	11.47 ± 0.02	324.6 ± 15.0	950.2 ± 4.7	338.4 ± 22.4
Caldas C-R	85 (280)	34.00 ± 0.15	6.38 ± 0.02	54.6 ± 1.3	777.4 ± 27.9	110.4 ± 12.1
La Unión LU-T	110 (292)	65.56 ± 0.28	34.50 ± 0.07	143.5 ± 5.5	869.5 ± 7.8	191.3 ± 31.8
Betania B-R	390 (242)	75.22 ± 1.35	42.37 ± 0.18	236.3 ± 9.1	1091.0 ± 17.3	227.6 ± 12.5
Apiary UN-T	72 (291)	22.11 ± 0.54	4.75 ± 0.01	33.9 ± 9.7	455.5 ± 7.8	40.9 ± 13.3
BHT (standard)				517.4 ± 0.1	884.9 ± 0.1	500.6 ± 5.5

[a]Units are mg of gallic acid/g of sample (mg GAE/g of EEP). [b]Units are mg of quercetin/g of sample (mg QE/g of EEP). Values are expressed as mean ± standard error of the mean (SEM) (n=3). [c]Antioxidant capacity in trolox equivalents (TE). [d]Antioxidant capacity in ascorbic acid equivalents. Values are expressed as mean ± standard error of the mean (SEM; n = 3).

B-R, Betania-scraping; C-T, Caldas-trap; C-R, Caldas-scraping; LU-T, La Unión-trap; UN-T, Apiary Universidad Nacional-Medellín-Trap.

evaluated in this work were collected by the methods of scraping (R) and plastic trap (T) during three periods – (1) June–August 2009; (2) August–November 2009; and (3) March–May 2010 – in three apiaries located in an area near to the municipalities of Zaragoza, El Bagre and Caucasia. The samples were named as Llanta Azul (LA), Doña María (DM) and Coco Hondo (CH), depending on the zone where they were collected (Table 7.5).

The $E^{1\%}_{1cm}$ analysis showed that the samples with the highest values were CH-2T, CH-2R, DM-2T, DM-2R, LA-2T and LA-2R. All the above propolis extracts had similar $E^{1\%}_{1cm}$ values ranging between 104 and 115. Moreover, it is important to highlight that all the samples with the greatest values were collected in the same season (period 2, dry season).

The same samples that showed the highest $E^{1\%}_{1cm}$ values had the highest values for phenolic compound content, with the exception of DM-2T and DM-2R that showed values even lower than samples LA-3T and LA-3R. In contrast to TPC, the samples collected in period 3 showed the greatest flavonoid content values, ranging from 2.49 to 3.52 mg of quercetin/mg EEP. Among the second period samples LA-2T was the sample with the greatest TFC value. With regard to flavanone content, all the samples showed similar values and the highest flavanone content values were shown by CH-2T and DM-2R, both with 0.26 ± 0.02 mg of pinocembrine/mg EEP. According to the sample collection method employed, we can appreciate that there is not a marked difference in the TPC, TFC and flavanone content values.

Propolis from the Atlántico Department

This department is located in the Caribbean region of Colombia that comprises seven more departments (Bolívar, Cesar, Córdoba, Guajira, Magdalena, San Andrés and Sucre) with approximately 182 municipalities. In the same direction, and depending on geographical accidents and ecosystems, the Caribbean region can also be divided into six geographical sub-regions. The Atlántico Department is located in the Magdalena river mouth region. We collected and analysed samples from four municipalities: Sabanalarga, Juan de Acosta, Santo Tomás and Galapa. To the best of our knowledge, there are no previous reports of chemical characterization and antioxidant activity evaluation of propolis from the Atlántico Department. The results obtained are outlined below.

The $E^{1\%}_{1cm}$ values of the EEP samples were between 26 and 79. All samples showed maximum wavelengths between 274 and 291 nm. These values are currently associated with the presence of phenolic compounds, flavonoids and caffeic acids, which have maximum wavelength values of between 240 and 350 nm.

The results summarized in Table 7.6 show TPC values ranging from 63.72 to 94.55 mg GAE/g EEP. Propolis samples from Juan de Acosta, Santo Tomás and Galapa showed a TPC content higher than propolis from Antioquia-Colombia (Palomino-García *et al.* 2009). It is worth noting that all the EEP from this region exceed the minimum requirement proposed by Brazilian legislation for phenolic compounds (50 mg GAE/g EEP). From all EEP tested, propolis from Sabanalarga was the sample with the lowest TPC value.

The TFC values for these samples were between 1.90 and 5.16 mg/g. Propolis from Santo Tomás showed the highest TFC (5.16 mg/g) and the second highest TPC value (86.63 mg/g). Moreover, it is relevant that EEP from Santo Tomás was the only sample that met TFC requirement parameters established by Brazilian regulation (5 mg/g) (Table 7.6). On the other hand, although propolis from Juan de Acosta showed the highest TPC value (94.55%), it had the lowest TFC value (1.90 mg/g). In addition, propolis samples with the lowest $E^{1\%}_{1cm}$ values, Juan de Acosta and Sabanalarga, showed the lowest values in TFC and TPC analysis, respectively.

7.4.5 Antioxidant activity of propolis samples from Colombia

Propolis from the Antioquia Department

SOUTH REGION. The antioxidant activity of these propolis samples was tested using

Table 7.5. Total phenolic content, total flavonoid content and antioxidant activity of EEP from different municipalities of North region of Antioquia Department, Colombia.

Sample	Total phenolic content (mg gallic acid/g EEP)	Total flavonoid content (mg quercetin/g EEP)	Total flavanone content (mg pinocembrin/g EEP)	DPPH• (μmol trolox/g of extract)	ABTS•+ (μmol trolox/g of extract)	FRAP (μmol trolox a.a./g extract)	$E^{1\%}_{1cm}$
CH-1T	33.41 ± 1.25	0.99 ± 0.04	0.08 ± 0.01	58.8 ± 0.0	405.5 ± 7.3	135.4 ± 0.4	58.03
CH-2T	93.30 ± 3.26	1.67 ± 0.01	0.26 ± 0.02	243.7 ± 0.0	1498.7 ± 37.3	n.d.	114.42
CH-3T	55.49 ± 0.90	2.66 ± 0.05	0.21 ± 0.02	77.7 ± 22.1	518.8 ± 13.6	230.2 ± 2.9	51.18
CH-1R	64.03 ± 3.96	0.92 ± 0.02	0.10 ± 0.01	46.1 ± 2.1	346.8 ± 19.1	100.2 ± 0.7	54.73
CH-2R	84.97 ± 0.42	0.99 ± 0.03	0.21 ± 0.01	164.7 ± 2.1	629.3 ± 10.9	n.d.	108.18
CH-3R	51.53 ± 0.42	3.91 ± 0.13	0.21 ± 0.02	87.2 ± 6.3	465.5 ± 13.6	206.2 ± 0.2	44.48
DM-1T	39.66 ± 0.00	1.12 ± 0.02	0.11 ± 0.01	82.5 ± 12.6	694.8 ± 2.7	155.4 ± 0.6	69.61
DM-2T	60.59 ± 3.33	0.94 ± 0.01	0.22 ± 0.02	169.5 ± 4.2	794.4 ± 9.1	n.d.	114.75
DM-3T	67.16 ± 3.54	2.74 ± 0.24	0.22 ± 0.01	77.7 ± 9.4	543.3 ± 16.4	241.0 ± 9.8	52.63
DM-1R	57.16 ± 4.17	1.12 ± 0.02	0.11 ± 0.01	77.7 ± 0.0	314.0 ± 5.5	135.3 ± 0.7	55.24
DM-2R	75.80 ± 1.81	0.89 ± 0.01	0.26 ± 0.02	147.30 ± 8.4	708.5 ± 14.6	n.d.	104.94
DM-3R	58.30 ± 0.14	2.49 ± 0.07	0.20 ± 0.02	101.5 ± 28.5	501.0 ± 10.0	269.1 ± 4.9	41.36
LA-1T	40.59 ± 2.08	0.87 ± 0.01	0.09 ± 0.01	79.3 ± 2.1	469.6 ± 8.2	60.1 ± 3.2	50.48
LA-2T	114.66 ± 1.46	2.15 ± 0.09	0.17 ± 0.02	771.8 ± 16.8	1767.5 ± 46.4	n.d.	113.96
LA-3T	93.93 ± 3.82	3.52 ± 0.09	0.19 ± 0.02	66.6 ± 5.2	510.6 ± 10.9	260.9 ± 1.09	35.70
LA-1R	57.68 ± 4.3	0.89 ± 0.02	n.d.	47.7 ± 2.1	316.8 ± 6.4	123.3 ± 0.8	66.91
LA-2R	82.36 ± 2.36	1.14 ± 0.02	0.15 ± 0.01	302.3 ± 4.2	1276.2 ± 57.3	n.d.	105.24
LA-3R	65.80 ± 2.57	2.60 ± 0.03	0.08 ± 0.01	145.70 ± 43.2	760.3 ± 8.2	308.8 ± 1.42	53.45

Samples were collected by scraping (R) or plastic trap (T) methods during three seasons: (1) June–August 2009; (2) August–November 2009; and (3) March–May 2010. The samples were named Llanta Azul (LA), Doña María (DM) and Coco Hondo (CH), depending on the zone where they were collected. n.d., not determined.

Table 7.6. Total phenolic content, total flavonoid content and antioxidant activity of ethanol extracts obtained from propolis collected in different municipalities belonging to the Atlántico Department of Colombia.

Sample	Specific absorbance of UV spectrum $E^{1\%}_{1cm}$ (λ)	Total phenolic content[a]	Total flavonoid content[b]	DPPH TEAC[c] ± SD (μmol/g)	ABTS TEAC[c] ± SD (μmol/g)	FRAP AEAC[d] ± SD (μmol/g)	ORAC (μmol TROLOX/ g of propolis)
Galapa	73.54 (290)	81.22 ± 10.01	3.36 ± 0.82	190.41 ± 6.90	1918.41 ± 44.45	321.27 ± 55.6	1964.80
Sabanalarga	29.04 (274.5)	63.72 ± 16.06	2.32 ± 0.04	65.14 ± 2.13	739.74 ± 14.51	112.05 ± 11.5	475.02
Santo Tomás	79.32 (291.5)	86.63 ± 4.38	5.16 ± 0.21	134.18 ± 3.54	1322.57 ± 4.66	262.65 ± 24.5	2211.94
Juan de Acosta	26.54 (283.5)	94.55 ± 21.06	1.90 ± 0.12	103.79 ± 2.48	1061.07 ± 19.55	191.12 ± 31.5	709.46
BHT (standard)	–	–	–	517.4 ± 0.1	884.9 ± 0.1	500.6 ± 5.5	–

[a]mg of gallic acid/g of sample (mg GAE/g EEP). [b]mg of quercetin/g of sample (mg QE/g EEP). Values are expressed as mean ± standard error of the mean (SEM) (n=3). [c]Antioxidant capacity in trolox equivalents (TE). [d]Antioxidant capacity in ascorbic acid equivalents. Values are expressed as mean ± standard error of the mean (SEM) (n=3).

three experimental models: DPPH, ABTS and FRAP. The results for DPPH and ABTS showed values ranging from 33.9 ± 9.7 to 324 ± 15.0 and 455.5 ± 7.8 to 17.3 μmol TE/g of EPP, respectively (Table 7.4). The samples of C-T and B-R showed the highest values in both antioxidant assays, and the extract with the lowest values was UN-T. The highest value in the DPPH and ABTS tests was for C-T and B-R extracts, respectively, even higher than the standard (BHT). It is worth mentioning that there is an appreciable difference between the radical scavenging activity values shown by C-T and C-R; this could be explained because, although both samples were collected in the same place, the method of collection employed was different.

As in DPPH and ABTS tests, the best values in the FRAP assay were shown by C-T and B-R but in both cases were lower than the standard (BHT). Again, the UN-T extract showed the lowest value in FRAP assay.

When we compared these results with TPC, TFC and $E_{1cm}^{1\%}$ values, a positive correlation can be appreciated. The samples, C-R and B-T, that displayed the highest values in the antioxidant activity assays, also showed the highest TPC, TFC and $E_{1cm}^{1\%}$ values. The opposite can be observed in the UN-T sample. Although more factors influence propolis antioxidant activity, the results are consistent with previous studies where a positive correlation between TPC and TFC content with a significant antioxidant activity is established (Gregoris and Stevanato, 2010).

NORTH REGION. The antioxidant capacity analyses were carried out with DPPH, ABTS and FRAP models. We want to emphasize some results from Table 7.5. The best antioxidant activity results in the three tests were obtained for LA-2T, DM-2T and CH-2T samples collected by trap in the second period (August–November 2009). This season is recognized in the zone as a dry time. The sample that showed the best value in DPPH antioxidant assay was LA-2T (771.8 ± 16.8), even three times more than the second one, CH-2T (243.7 ± 0.0). Similarly, LA-2T had the highest scavenging activity against the ABTS cation, followed by CH-2T. Unfortunately, a FRAP analysis of the most active samples was not done.

A second important factor to consider is the method of collection. A comparison between samples collected in the same period (i.e. CH-1T and CH-1R) by the two methods (scraping and plastic trap) clearly show that the highest antioxidant activity values are for the samples that were collected by plastic trap.

These results are in line with the values obtained in TPC. For this case, the sample LA-2T that presented the highest DPPH, ABTS and FRAP values also showed the highest phenolic content.

Propolis from the Atlántico Department

In addition to the three antioxidant analyses carried out with the samples of Antioquia, we also analysed the propolis samples of Atlántico with the ORAC assay. The results obtained with these samples are outlined in Table 7.6.

The propolis from Galapa was the most active extract in the DPPH radical test (190.41 μmol TE/g EEP), followed by Santo Tomás (134.18 μmol TE/g EEP) and Juan de Acosta (103.79 μmol TE/g EEP). The lowest scavenging radical DPPH activity was shown by Sabanalarga (65.14 μmol TE/g EEP).

A similar trend to that observed in the DPPH analysis was shown in the discoloration of the blue radical cation ABTS and FRAP assays because, in both tests, the propolis from Galapa was the most active and the propolis from Sabanalarga the lowest. None of the samples tested presented higher antioxidant capacity values than the standard.

In the ORAC analysis the highest values were shown by extracts from Santo Tomás (2211.94 μmol Trolox/g propolis) and Galapa (1964.80 μmol Trolox/g propolis), respectively. Following the tendency, the lowest value in the ORAC test was Sabanalarga's sample (475.02 μmol Trolox/g propolis).

Furthermore, extracts of propolis collected in Galapa, Santo Tomás and Juan de

Acosta presented the highest values in TPC and TFC (Table 7.6). The sample from Juan de Acosta showed the highest TPC value but the lowest in TFC; this could be because flavonoids are not the only compounds with antioxidant capacity and maybe another type of phenolic substance could be involved (Huang *et al.*, 2005; Mohammadzadeh *et al.*, 2007).

7.4.6 Final comments on TPC, TFC and antioxidant activity of Colombian propolis

Almost all samples, with the exception of CH-1T, DM-1T and LA-1T, meet the Brazilian regulation for TPC (50 mg GAE/g EEP). It is important to note that the samples that do not comply with TPC minimum values were collected in the same season using the scraping method. A different situation is observed with respect to TFC analysis: all the samples obtained from the Southern region of Antioquia comply with Brazilian values for TFC (5 mg/g of EEP) but none of the samples of the Northern region reached the minimum value for TFC. The highest values of TFC for samples of this region were CH-3R (3.91) and LA-3T (3.52). From the group of samples of the Atlantic region, only the sample from Santo Tomás meets the TFC standard value.

When we analyse the antioxidant activity shown by the extracts, a strong relationship between TPC values, TFC values and antioxidant capacity can be observed. The propolis extracts (Betania, LA-2T, DM-2T, CH-2T, Galapa, Santo Tomás) that showed the highest TP and TF content were the samples with the best DPPH, ABTS and FRAP values. As mentioned before, phenolic and flavonoid compounds are considered to contribute to the biological and pharmacological activities shown by this resinous substance and our results are in agreement with that.

Additionally, we can visualize some factors, referred to in many studies, that can influence the chemical composition of propolis and therefore the biological activities that they present. On the basis of our results it is evident that the variability in chemical composition is strongly influenced by geographical zones where the propolis samples were collected and this factor is closely related to the vegetation used by bees to produce propolis.

On the other hand, differences in composition are clearly seen when the samples were collected in the dry season with respect to other periods of collection. Moreover, some differences in composition and antioxidant activity can be appreciated depending on the method of collection. The samples that were collected by the plastic trap method showed the highest phenolic and flavonoid content, and showed the best results in the antioxidant methods.

Finally, although most of the activities are attributed to phenolic compounds, there are, particularly in tropical regions, other classes of metabolites that could be responsible, i.e. terpenes. As can be noted, there are several factors influencing the metabolic profile of propolis. For standardization of Colombian propolis, as an active principle and cosmetic ingredient, there is a need to carry out further chemical and biological analysis with samples from other departments to create a regulation that represents Colombia's scene.

7.5 Concluding Remarks

There are many studies on the chemical composition and biological activities of propolis from different regions around the world. Most of them have essentially established a correlation between the pharmacological properties and the chemical composition. Additionally, the variability of compounds found in propolis samples is strongly influenced by the surrounding flora. Although Colombia is a tropical country with a great biodiversity, beekeeping is a poorly developed sector and this leads to a general lack of knowledge with regard to the characterization of products from the beehive. Furthermore, our country does not have an official regulation to establish the quality of the propolis. For those reasons, a priority is to

direct efforts towards establishing biological properties (antioxidant and antimicrobial), chemical composition, botanical origin of the species employed by bees and minimum quality parameters of propolis samples from different regions of Colombia. With this data it will be more feasible to standardize the process of propolis production, which will allow the development of innovative products with this resinous substance. Finally, the study of propolis from regions not previously explored will be attractive for researchers trying to find new bioactive molecules.

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8 Chilean Plants as a Source of Polyphenols

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8.1 Traditional Uses of Native Medicinal Plants from Chile

The Chilean flora represents an important genetic resource, especially considering its high degree of endemism. Central Chile is considered a biodiversity hotspot (Myers *et al.*, 2000). Of the 5105 higher plants that form part of the Chilean flora, 45.8% are endemic (Marticorena, 1990).

The native flora was used by pre-Hispanic inhabitants for various purposes, such as food, fuel, religious ceremonies, decoration, dyeing and medicine. The tradition of using plants for medicinal purposes by native populations in Chile was recorded in the archives of the settlers who, in turn, enriched them with the contribution of medicinal plants from Europe and other regions (Massardo and Rossi, 1996). Naturist medicine books show that the medicinal use of plants native to Chile covers a wide spectrum of disease and healing practices (Rozzi and Massardo, 1994a,b). This knowledge, based on ethnobotany and ethnopharmacology, has been recognized both nationally and internationally during the last three decades by many authors (Montes and Wilkomirsky, 1987; Graham *et al.*, 2000; Muñoz *et al.*, 2001; Evans, 2002; Montenegro, 2002; Jagtenberg and Evans, 2003; Molares and Ladio, 2009).

In the early 1990s a group of experts convened by the Chilean Health Ministry reported that the use of local medicinal herbs lacked appropriate technical definitions to differentiate between medicinal plants and herbal teas, and that there were significant deficiencies in their production process. Following this, a multidisciplinary group of both public and private advocates created the first registry of medicinal plants used empirically by the Chilean population. Only 28% of these plants had any scientific validation (Minsal, 2009). Since 2007 The Chilean Health Ministry (Minsal, 2007, 2008) has approved two lists (103 species) of plants that are considered traditional herbal medicines (THM). This regulation requires that product labels include the term used by popular custom, as part of cultural tradition. Moreover, manufacturers are obliged to designate the properties of their products, as these lists are recognized as official. Table 8.1 outlines Chilean medicinal plants, the parts used and their effects, in order to analyse compounds that could be responsible for these medicinal effects.

Many common names of plants described in Table 8.1 derive from Mapudungun (the

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Table 8.1. Chilean plants reported as traditional herbal medicine, parts used and their effects. (Adapted from Minsal, 2007, 2008.)

Species	Common name	Family[a]	Distribution[a]	Parts used	Effects
Acaena splendens Hook et Arn	Cepa caballo-Amores secos	*Rosaceae*	Chile Argentina	Leaves	Anti-inflammatory Diuretic Purifier
Acantholippia deserticola (Phil.) Moldenke	Rica-Rica-Kore	*Verbenaceae*	Chile Argentina	Stems Leaves Flowers	Antiseptic Antispasmodic
Aloysia citriodora Paláu (syn. *A. triphylla*)	Cedrón	*Verbenaceae*	Chile Argentina Bolivia Brazil Uruguay Paraguay	Stems Leaves	Antibacterial Antispasmodic Carminative Mild sedative
Aristotelia chilensis (Mol.) Stunz	Maqui	*Elaeocarpaceae*	Chile Argentina	Leaves Fruits	Analgaesic Anti-inflammatory Antiseptic Antispasmodic Astringent
Buddleja globosa Hope	Matico-Pañil	*Scrophulariaceae*	Chile Peru Argentina	Leaves	Analgaesic Antifungal Anti-inflammatory Cicatrizant
Calceolaria thyrsiflora Grah.	Hierba Dulce-Palo dulce	*Calceolariaceae*	Chile	Leaves	Anti-inflammatory Astringent Cicatrizant Diuretic
Centaurium cachanlahuen B.L. Rob.	Cachalagua-Cachan-Lawen	*Gentianaceae*	Chile Argentina	Entire plant, especially leaves and stems	Antihypertensive Appetite stimulator Febrifuge Hypoglycaemic Purifier
Cestrum parqui L'Herit	Palqui-Parqui	*Solanaceae*	Chile Argentina	Stems without bark Leaves	Anti-inflammatory Cicatrizant Febrifuge

Continued

Table 8.1. Continued.

Species	Common name	Family[a]	Distribution[a]	Parts used	Effects
Chenopodium ambrosioides L.	Paico	*Chenopodiaceae*	Chile Other countries	Leaves	Anthelmintic Anti-inflammatory Antispasmodic Emmenagogue
Cuscuta chilensis Ker-Gawl	Cabello de ángel	*Convolvulaceae*	Chile	Entire plant	Anti-inflammatory Diuretic
Drimys winteri J.R. et G. Forster	Canelo-Voigue	*Winteraceae*	Chile Argentina	Leaves Bark	Analgaesic Anthelmintic Antibacterial Antispasmodic Cicatrizant
Ephedra chilensis C. Presl	Pingo-Pingo	*Ephedraceae*	Chile Argentina	Aerial parts	Diuretic Purifier
Equisetum bogotense H.B. et Kunth	Yerba de la plata	*Equisetaceae*	Chile	Branches	Anti-inflammatory Cicatrizant Diuretic Haemostatic
Fabiana imbricata R. et P.	Pichi-Pichi romero	Solanaceae	Chile Argentina	Branches	Antiseptic Digestive Diuretic
Flaveria bidentis (L.) O. Kuntze	Contrayerba-Mata Gusanos-Dalal	*Compositae*	Chile Argentina Bolivia Brazil Paraguay Uruguay	Leaves Branches	Anthelmintic Emmenagogue Laxative Sudorific
Fuchsia magellanica Lam.	Chilco-Fucsia	*Onagraceae*	Chile Argentina	Leaves Flowers	Diuretic Emmenagogue Febrifuge
Geum andicola (Phil.) Reiche	Yerba del clavo-Leliantu	*Rosaceae*	Chile Argentina	Rhizome Roots	Expectorant Purifying Sudorific

Gunnera tinctoria (Mol.) Mirb	Nalca-Pangue	*Gunneraceae*	Chile Argentina	Root Stem Leaves	Astringent Febrifuge Haemostatic
Haplopappus spp.	Baylahuén	*Compositae*	Chile Argentina	Leaves Stems	Antiseptic Digestive
Lampaya medicinalis F. Phil.	Lampayo	*Verbenaceae*	Chile	Leaves	Purifier
Laretia acaulis (Cav.) Gill. et Hook.	Llareta-Yareta	*Apiaceae*	Chile Argentina	Entire plant	Anti-inflammatory Hypoglycaemic Purifier
Libertia sessiliflora (Poepp.) Skottsb	Triqui-Triqui-Huilmo	*Iridaceae*	Chile	Entire plant	Digestive Diuretic Emmenagogue Febrifuge Laxative
Lomatia hirsuta (Lam) Diles ex Macbr.	Radal	*Proteaceae*	Chile Argentina Peru Ecuador	Leaves	Anti-inflammatory Antimicrobial Bronchodilator
Luma chequen (Mol.) A. Gray	Arrayan-Chequén	*Myrtaceae*	Chile	Branches with leaves	Anti-inflammatory Purifier
Margyricarpus pinnatus Kuntze.	Sabinilla-Perlilla	*Rosaceae*	Chile Argentina Brazil Uruguay	Leaves Stems Branches	Antispasmodic Diuretic Purifier
Maytenus boaria Mol.	Maitén	*Celastraceae*	Chile Argentina Bolivia Brazil	Leaves Branches Seeds	Anti-inflammatory Febrifuge Purifier
Muehlenbeckia hastulata I.M. Johnst.	Quilo-Mollaco-Voqui	*Polygonaceae*	Chile Argentina	Entire plant	Astringent Diuretic Purifier
Otholobium glandulosum (L.) Grimes	Culén	*Leguminosae*	Chile Peru	Leaves Stems Bark Flowers	Antibacterial Astringent Cicatrizant Febrifuge

Continued

Table 8.1. Continued.

Species	Common name	Family[a]	Distribution[a]	Parts used	Effects
Peumus boldus Mol.	Boldo	*Monimiaceae*	Chile	Leaves	Anti-inflammatory Digestive Laxative Liver protector
Polypodium feuillei Bertero	Yerba del lagarto-Calaguala	*Polypodiaceae*	Chile	Leaves Stems Rhizome Roots	Expectorant Purifying Sudorific
Porlieria chilensis I.M. Johnst	Guayacán-Palo Santo chileno	*Zygophyllaceae*	Chile	Branches Bark	Antibacterial Anti-inflammatory Balsamic Purifier Sudorific
Pseudognaphalium viravira (Mol.) A. Anderb.	Vira-Vira	*Compositae*	Chile	Entire plant	Antibacterial Expectorant Febrifuge Sudorific
Quillaja saponaria Mol.	Quillay	*Quillajaceae*	Chile	Bark	Emollient Expectorant Sudorific
Quinchamalium chilense Mol.	Quinchamalí	*Santalaceae*	Chile Argentina Bolivia	Stems Leaves Flowers	Anti-inflammatory Cicatrizant Diuretic Purifier
Salix humboldtiana Willd. Salix chilensis Mol.	Sauce amargo-Sauce chileno	*Salicaceae*	Chile Argentina Bolivia Brazil Uruguay	Bark Leaves	Analgaesic Anti-inflammatory Astringent Febrifuge

Schinus areira L. (syn. *S. molle* L.)	Molle	*Anacardiaceae*	Chile Argentina Bolivia Brazil Uruguay Paraguay Peru Mexico Ecuador Colombia	Leaves Bark	Antibacterial Antifungal Anti-inflammatory Antispasmodic Cicatrizant Emmenagogue
Senecio fistulosus Poepp. ex Less	Hualtata-Lampazo	*Compositae*	Chile Argentina	Leaves	Cardiotonic Diuretic
Senna stipulacea (Aiton) Irv. et Barneby	Quebracho Blanco-Sen Chileno	*Leguminosae*	Chile	Stems	Laxative
Solanum ligustrinum Lodd. (*S. crispum* and *S. gayanum*, included)	Natre	*Solanaceae*	Chile Argentina	Leaves Stems	Febrifuge Hypoglycaemic
Tristerix corymbosus (L.) Kuijt	Quintral	*Loranthaceae*	Chile Argentina	Leaves Flowers Branches	Anti-inflammatory Cholesterol-lowering Cicatrizant Haemostatic Mild sedative

[a]PROflora and Royal Botanic Garden Edinburgh (2010) and Instituto de Botánica Darwinion (2010).

language of the Mapuche, an indigenous group from Chile). Villagran (1998) indicates that most Mapuche plant names refer to morphological (51.1%), ecological (19.3%) and utilitarian (21%) relationships. The morphological relationships take into account properties of the plant itself as well as morphological similarities with other plants, animals and objects. The ecological relationships refer both to habitat properties and plant sociology as well as interactions with animals, mainly birds. Of the names based on utilitarian relationships, half of these express medicinal properties that are specific to the plants.

The Mapuche medicinal flora holds a vast, rich body of knowledge learned and transmitted within the culture throughout space and time (Estomba *et al.*, 2006; Lozada *et al.*, 2006). For example, Molares and Ladio (2009) quantitatively reviewed information on Mapuche ethnobotany, published for Argentina and Chile, that considered rural communities distributed throughout an area extending between approximately 37° and 46° (South Latitude). These communities were established in different phytogeographical provinces. Among these, communities in Central Chile were studied. These authors recorded 505 species, of which 304 (60%) were native and 196 (39%) exotic. This review covered more native species than the lists of THM (Table 8.1), which considered only 39% of Chilean plants. This confirms the significant contribution of exotic plants to the THM knowledge of Chile. On the other hand, part of this traditional knowledge is being lost due to the high migration of population from rural to urban areas. Also some reports (FAO, 2008) by Chilean public institutions showed that only about 30% of Chilean plants have any known use.

The medicinal effects related to Chilean native plants in Table 8.1 are very extensive (i.e. 30 different effects). The most common are: anti-inflammatory (45% of species), purifier (32.5% of species), diuretic (30% of species), febrifuge (25% of species) and antimicrobial/antibacterial and antifungal (22.5% of species). These effects could be attributed to plant natural products (PNPs) that have shown many types of biological activities (BAs) mainly in *in vitro* studies. Terpenes (isoprenoids) have shown antimicrobial activity against some Gram-positive and Gram-negative bacteria (Potduang *et al.*, 2007; Poblete *et al.*, 2009; Popova *et al.*, 2009), human pathogenic fungi (Popova *et al.*, 2009) and protozoa (Olagnier *et al.*, 2007; Pinheiro *et al.*, 2009). The wide use of plant essential oils (i.e. mixture of diterpenes, sesquiterpenes and polyphenols) as bactericidals, virucidals, fungicidals, antiparasiticals and insecticidals has been carefully compiled by Bakkali *et al.* (2008). Terpenes also have analgaesic (Corvalán *et al.,* 2008), anti-hyperglycaemic and anti-inflammatory (Paduch *et al.*, 2007) effects. Alkaloids exhibit antiviral, antimalarial, antioxidant, anti-inflammatory (Okwu and Uchenna, 2009), antifungal (Kuta, 2008; Okwu and Uchenna, 2009) and anti-tumour activities (Ingrassia *et al.*, 2008). Alkaloids also have antimicrobial activity against plant-parasitic nematodes (Thoden and Boppre, 2010). Polyphenols have cardioprotective (Cale *et al.*, 2010; Xia *et al.*, 2010), antioxidant (Bouaziz *et al.*, 2009; Janicsak *et al.*, 2010; Xia *et al.*, 2010), antimicrobial (Bouaziz *et al.*, 2009; Xia *et al.*, 2010), anticancer and anti-inflammatory properties (Xia *et al.*, 2010). Much research of the BA of plants considers extracts (e.g. acetone, methanol and water extracts). This means that in plant extracts (PEs) that contain a mixture of terpenes, alkaloids and polyphenols (Jimoh *et al.*, 2010) or compounds that are soluble in these different solvents, the potent BA could be more attributed to a 'cocktail' of PNPs rather than to individual organic compounds.

8.2 Scientific Evidence Supporting Biological Activity Associated with the Presence of Polyphenols in Chilean Plants

Several thousand molecules having a polyphenol structure (i.e. several hydroxyl groups on aromatic rings) have been identified in higher plants. These compounds are

classified into different groups. On the basis of the number of phenol rings that they contain and of the structural elements that bind these rings to one another, polyphenols can be divided into phenolic acids, flavonoids, stilbenes and lignans. The flavonoids, which share a common structure consisting of two aromatic rings (A and B) that are bound together by three carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into six subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (catechins and proanthocyanidins). In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids and with one another (Manach *et al.*, 2004). Flavonoid properties related to their antioxidant (Chung *et al.*, 2009; Guabiraba *et al.*, 2010; Justino and Vieira, 2010; Sadowska-Woda *et al.*, 2010; Zhang *et al.*, 2010) and anti-inflammatory activity have been extensively reported during recent years. Inflammation and oxidative stress are associated with several diseases, and flavonoids have been intensively investigated for anti-inflammatory and antioxidant properties.

Many plants described in lists of THM do not have any scientific evidence supporting their BA or traditional medicinal uses (e.g. *Centaurium cachanlahuen, Ephedra chilensis, Lampaya medicinalis, Libertia sessiliflora, Otholobium glandulosum* and *Polypodium feuillei*) according to an exhaustive search in Web of Science® (provided by Pontificia Universidad Católica of Chile). On the other hand, there is some scientific evidence for other kinds of PNP, such as terpenoids (e.g. *Laretia acaulis, Luma chequen, Margyricarpus pinnatus* and *Maytenus boaria*) and alkaloids (e.g. *Peumus boldus*). Therefore, scientific evidence supports the BA of some species that have not been described in THM lists (e.g. *Gevuina avellana, Laurelia sempervivens, Satureja parvifolia* and *Ugni molinae*). Within this search, different BA could be attributed to the presence of polyphenols in Chilean plants. Table 8.2 summarizes information from scientific articles published between 2000 and 2010. Previous scientific publications have been compiled by Muñoz *et al.* (2001) and Montenegro (2002).

Antimicrobial (i.e. antifungal, antibacterial and nematicidal), analgaesic and anti-inflammatory are the main BAs studied in Chilean plants that could be related to the presence of polyphenols (Table 8.2). Among this research *Buddleja globosa* has been the most important species studied. Some polyphenols have been described in this research – most of them are flavonoids. Nevertheless, some of these articles did not identify the polyphenols contained in plant extracts. For this reason, it is very difficult to establish a clear relationship between certain polyphenols identified in these plants and the type of BA described. Important exceptions were Guglielmone *et al.* (2002, 2005) and Céspedes *et al.* (2006) whose research isolated some flavonols (quercetine types) and five lignans (secoisolariciresinol, pinoresinol, eudesmin, lariciresinol and lariciresinol-4-methyl ether) from *Flaveria bidentis* and *Araucaria araucana,* and demonstrated their anticoagulant and antimicrobial activity, respectively.

8.3 Polyphenols and Antioxidant Capacity in Chilean Plants

Given that the majority of the BAs of polyphenols may be also attributed to their antioxidant capacity (AC), the determination of total phenol content (TPC) and AC of a species is currently a scientifically valid tool to determine its antioxidant and pharmacological potential. Since the 1990s studies have been published about the presence of polyphenols and the AC of Chilean plants. These articles consider chromatographic techniques (HPLC) connected to different types of detectors (UV, DAD and ESI-MS) for the identification of polyphenols. Various *in vitro* methods have also been used for the quantification of TPC (Folin–Ciocalteau method), total antioxidant capacity (TAC; differential pH method) and AC (e.g. ferric ion reducing antioxidant power [FRAP], total radical trapping

Table 8.2. BA of Chilean plants that could be related to polyphenols.

Activity	Causal agent	Species	Type of extract/part used	Polyphenols identified in the extracts	References
Analgaesic	na	*M. hastulata*	Sequential ethanol, hexane, dichloromethane aerial part and the root of the plant	Epicatechin, emodin-8-glycoside and rutin	Erazo *et al.*, 2002
	na	*B. globosa*	Sequential hexane, dichloromethane, methanol and total methanol leaf extract	Verbascoside and luteolin 7-O-glucoside (flavonoids)	Backhouse *et al.*, 2008a; Backhouse *et al.*, 2008b
Antibacterial		*C. thyrsiflora*	Flower and leaf infusion	Gallic acid	Bravo *et al.*, 2005
	Citrobacter sp., *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus*	*A. araucana*	Five lignans isolated from heartwood, methanol heartwood extract	Secoisolariciresinol, pinoresinol, eudesmin, lariciresinol, and lariciresinol-4-methyl ether, and other phenols	Céspedes *et al.*, 2006
	Staphylococcus aureus	*F. bidentis*	Chloroform aerial part extract	ni	Bardon *et al.*, 2007
Anticoagulant	na	*F. bidentis*	Flavonoids from leaves	Sulfated quercetin 3,7,3',4'-tetrasulphated and quercetin 3-acetyl-7,3',4'-trisulphate	Guglielmone *et al.*, 2002; Guglielmone *et al.*, 2005
Antifungal	*Mucor miehei, Paecilomyces variotii, Ceratocystis pilifera, Trametes versicolor*	*A. araucana*	Five lignans isolated from heartwood	Secoisolariciresinol, pinoresinol, eudesmin, lariciresinol, and lariciresinol-4-methyl ether	Céspedes *et al.*, 2006
	Aspergillus fumigatus, A. niger, Botryodiplodia theobromae, Cladosporium cladosporioides, Fusarium oxysporum, Helminthosporium oryzae, Macrophomina phaseolina, Phythium debaryanum and Sclerotium rolfsii	*C. ambrosioides*	Fresh plant essential oil	ni	Kumar *et al.*, 2007
	Botrytis cinerea Pers	*Q. saponaria*	Ethanolic and aqueous shoot extract	Chlorogenic, caffeic, vanillic and salicylic acids, and scopoletin	Ribera *et al.*, 2008

Anti-inflammatory	na	*A. splendens*	Methanol (column fractionation) whole plant extract	Epicatechin, tiliroside, 7-O-acetyl-3-O-D-glucosyl-kaempferol and 7-D-glucosyloxy-5-hydroxy-chromone	Backhouse *et al*., 2002
	na	*B. globosa*	Sequential hexane, dichloromethane, methanol and total methanol leaf extract	Verbascoside and luteolin 7-O-glucoside (flavonoids)	Backhouse *et al*., 2008b
	na	*A. chilensis*	Ethanol, acetone, ethyl acetate fruit extract	Anthocyanins, other flavonoids and phenolic acids	Céspedes *et al*., 2010a
Antiplasmodial (antimalarial)	na	*B. globosa* and *S. parvifolia*	Methanol and aqueous plant extract	ni	Debenedetti *et al*., 2002
Antithrombin	na	*G. tinctoria*	Methylene chloride - methanol plant extract	ni	de Medeiros *et al*., 2000
Cardioprotective	na	*A. chilensis*			Céspedes *et al*., 2008
Nematicidal	*Meloidogyne hapla*	*B. globosa, D. winteri*, G. *avellana, Lonicera sempervirens, Luma apiculata, M. boaria* and *U. molinae*	Dry ground leaves	ni	Böhm *et al*., 2009

na, not applicable; ni, unidentified.

antioxidant parameter [TRAP], total antioxidant response [TAR], thiobarbituric acid reactive substances [TBARS], diphenyl-1-picrylhydrazyl [DPPH] and oxygen radical absorbance capacity [ORAC] methods). It is important to understand that these methods do not give the same results because different radicals are used. In some cases, a plant extract that shows the best AC with a specific method will not necessarily show the best AC with another method. For this reason, there is no single method appropriate to measure the total efficacy of an antioxidant. In assessing antioxidant activity it is important to concentrate on the mechanism of action, because there are many ways to generate free radicals, many ways in which a free radical can be quenched by an antioxidant, many factors that can influence this reaction and an almost infinite number of possible targets of the free radicals (Parr and Bolwell, 2000). Despite wide usage of these chemical AC methods, their ability to predict *in vivo* activity has not been clearly demonstrated (Mermelstein, 2008). The current challenge is the study of AC in *in vivo* models and polyphenols bioavailability in the human body.

8.3.1 Plant infusions

Plant infusions have been deeply rooted among South Andean indigenous cultures such as Aymara, Quechua (Villagrán *et al.*, 2003) and Mapuche (Montes and Wilkomirsky, 1987). Additionally, drinking an herbal infusion after a meal is a very common custom in Chile (Montes and Wilkomirsky, 1987; Montenegro, 2002). Vogel *et al.* (2005) studied the antioxidant properties (lipid peroxidation in erythrocytes and DPPH) of four Chilean *Haplopappus* species commonly named Baylahuén (*Haplopappus baylahuen, Haplopappus taeda, Haplopappus multifolius* and *Haplopappus remyanus*) to clarify their medicinal effects, because some confusion exists about the taxonomic identification of these species. *H. baylahuen* showed the lowest AC. In DPPH analyses an infusion (10%) of *H. baylahuen* also showed the lowest (661 μTE; TE, Trolox equivalents) inhibiting activity of free radicals, whereas *H. remyanus* showed the highest (3784 μTE). The chemical characterization of the studied species showed important levels of flavonoids and coumarins, with flavonoids predominating in *H. taeda*, coumarins in *H. multifolius* and both of them in *H. baylahuen* and *H. remyanus*.

Avello and Pastene (2005) evaluated the AC (ORAC method) in plasma before and after the intake of *Ugni molinae* leaf infusion (1%). In this study healthy volunteers (normal-lipidemic and non-diabetic) drank this infusion twice a day for 3 days. The results indicated a significant increase (from 2258 to 3108 μM TE/l) in the AC in plasma from volunteers. A similar study (Avello *et al.*, 2008) evaluated the AC (TBARS method) in plasma before and after the intake of *Aristotelia chilensis* leaf infusions (1%). The TPC in the infusion was 0.074 mM GAE (gallic acid equivalents). In this study healthy volunteers (no smoking and body mass index within the normal range) drank this infusion twice a day for 3 days (doses described by ethno-medicine for the treatment of various illnesses). The results showed an average increase of the AC at 24 h observed by means of TBARS (30.27%).

Rojo *et al.* (2009) studied the antioxidant capacities of 12 herbal teas widely consumed by South-American populations. The plant species (*Adesmia melanthes, Senecio nutans, Chuquiraga atacamensis, Fabiana densa, Fabiana squamata, Lampaya medicinalis, Azorella compacta, Baccharis tola, Opuntia ignescens, Acantholippia deserticola, Parastrephia lepidophylla* and *Parastrephia lucida*) were collected fresh in the province of Colchane located at 3500 m above sea level, in the Chilean administrative region of Tarapacá. Herbal infusions were prepared from plants grown under similar climatic conditions and were assessed for their TPC and *in vitro* AC (Trolox equivalent antioxidant capacity [TEAC]-DPPH, TEAC-crocin and FRAP). Although the results indicated TPC between 37 mg GAE/l (*B. tola*) and 314 GAE/l (*F. densa*), by far the most potent antioxidant species according to the FRAP assay were

O. ignescens (51,800 μmol TE/l) and *A. deserticola* (56,200 μmoL TE/l). This was not the same pattern observed when analysed by the TEAC-DPPH and TEAC-crocin methods.

Speisky *et al.* (2006) evaluated the AC of 13 herbal teas (herb bags) distributed in the Chilean market that were derived from five Chilean plants (*Haplopappus baylahuen, Peumus boldus, Buddleja globosa, Chenopodium ambrosioides* and *Aloysia citriodora*). On the basis of the TEAC assay, 150 ml of tea prepared from *H. baylahuen* and *P. boldus* would be equivalent to around 200 mg of Trolox. Infusions from *H. baylahuen* and *P. boldus* were also found to be particularly potent in quenching HClO. In the ONOO method *H. baylahuen* and *B. globosa* showed the highest activities. In a similar study (Poblete *et al.* 2009) TPC and ORAC values of *A. citriodora* herbal teas were determined. For this study the TPC of the infusions was 2.67 mM GAE and ORAC values were 0.20 and 9.45 mM GAE (ORAC-PGR and ORAC-FL, respectively).

Wernert *et al.* (2009) studied polyphenols of infusions and decoctions of *A. citriodora.* The results showed the presence of caffeic acid derivatives (hydroxycinnamic acids) and flavonoids (flavones). The TPC was 51.9 and 51.5 mg tannic acid equivalent (TAE)/g (dry weight), flavones were 20.8 and 20.7 mg TAE/g (dry weight), and total tannin content (TTC) was 6.0 and 8.9 mg TAE/g (dry weight) for infusions and decoctions, respectively.

Simirgiotis and Schmeda-Hirschmann (2010) identified phenolic constituents of *P. boldus* leaf infusions by HPLC-DAD and HPLC-MS. The phenolic constituents identified were mainly proanthocyanidins and flavonol glycosides (41 compounds were detected in male and 43 compounds in female leaf samples). Nine quercetin glycosides, eight kaempferol derivatives, nine isorhamnetin glycosides, three phenolic acids, one caffeoylquinic acid glycoside and 21 proanthocyanidins were identified. Isorhamnetin glucosyl-di-rhamnoside was the most abundant flavonol glycoside in the male samples, whereas isorhamnetin di-glucosyl-di-rhamnoside was the main phenolic compound in female leaf infusion. The authors suggested that the medicinal properties reported for this popular infusion should be attributed not only to the presence of catechin and boldine but also to several phenolic compounds with known antioxidant activity. The results obtained in this research could be a valuable contribution to the validation of the traditional uses of Chilean plant leaf infusions. The TPC and AC could be employed as a measure of the average quality of the polyphenols present in Chilean plant infusions in a similar way as the validation and promotion of other plant species such as green and black tea (*Camellia sinensis*).

8.3.2 Plant extracts

Peumus boldus is one of the most studied Chilean plants for its PNP. Traditionally, research was focused on the presence of boldine (alkaloid) and their BA. During the past decade research on the importance of polyphenols in *P. boldus* PE has been published. Schmeda-Hirschmann *et al.* (2003) studied the AC (DPPH method) of hot water extracts of *P. boldus* leaves. Method-guided isolation (HPLC-UV) also led to the active compounds. The IC_{50} values for catechin and boldine in the lipid peroxidation test were 75.6 and 12.5 μg/ml, respectively. On the basis of dry starting material, the catechin content in the crude drug was 2.25%, whereas the total alkaloid calculated as boldine was 0.06%. The activity of boldine was six times higher than catechin in the lipid peroxidation method. However, the mean catechin total alkaloid content ratio was 37:1. The relative concentration of alkaloids and phenolics in *P. boldus* leaves and their activity suggested that the free-radical scavenging effect was mainly due to catechin and flavonoids and that AC is mainly related to the catechin content. Similar results were obtained by Quezada *et al.* (2004) who determined the contribution of the flavonoid and other crude fractions from *P. boldus* to the AC (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid [ATBS] method) of leaf ethanolic extracts. The AC came mainly

from the flavonoid fraction (44.1%) followed by the alkaloid fraction (15.6%), with catechin and boldine being the main contributors of the AC of these two fractions (60.9% and 35.6% of the total activity, respectively).

Morales *et al.* (2008) evaluated the TPC and AC (DPPH method) of 50% aqueous-ethanol extract from *Acantholippia deserticola.* The TPC of the extract was 725 mg GAE/g (dried weight). The results showed that the IC_{50} value of the extract was 18 μg/ml.

Avello *et al.* (2009) evaluated the TPC and AC (DPPH method) of leaf extracts from *Aristotelia chilensis* and *Ugni molinae*. For measurements the extracts were standardized in GAE through the Folin–Ciocalteu method, which is a form of standardized extracts in a chemical marker. The TPC were 0.031–0.035 M GAE for *U. molinae* leaf extracts (MeOH 100% and MeOH/H_2O [60:40], respectively) and 0.032–0.040 M GAE for *A. chilensis* (H_2O 100% and EtOH/H_2O [60:40], respectively).

Dade *et al.* (2009) assessed the AC (ABTS and DPPH), TPC and flavonoid content (FV) of 21 extracts obtained from Argentinian native plants, among which there were some plants also native to Chile (*Lippia turbinata, Satureja parviflora, Aloysia triphylla, Xanthium spinosum, Flaveria bidentis* and *Geoffroea decorticans*). All extracts were able to bleach the radicals in the range of 0.03–4.48 μM TE/mg (dry extract). *S. parvifolia* extracts exhibited the highest scavenging activity for both DPPH radical (1.48 μM TE/mg [dry extract]) and ABTS radical (3.20 μM/TE mg [dry extract]). There was a significant correlation between the TP and FV content and the AC (P <0.001). This is concordant with other studies in plants from the *Labiatae* family, which are distinguished for their TPC and AC (Rossi *et al.*, 2009).

8.3.3 Edible fruit extracts

The main Chilean fruits studied for their polyphenols and AC are fruits recognized as berries. In botanical terms, a berry is a fruit with many seeds, mesocarp and endocarp flesh that evolve from a flower with a superior ovary (Bowling, 2000). Therefore, in strictly botanical terms, few of the fruits called berries are true berries. Nevertheless, the use of the term berry is widespread in scientific and commercial fields (Seeram, 2008). Interest in the study of Chilean native berries reflects a global trend of the search for fruit and new raw materials with high levels of antioxidants.

Aristotelia chilensis (maqui) fruits are significant for their TPC and AC. These fruits have greater TPC and AC (FRAP, TAR and TRAP methods) than other species recognized by their high polyphenol contents such as *Vaccinium corymbosum* L. (blueberries), *Rubus* spp. (blackberries), *Vaccinium macrocarpon* Ait (cranberries), *Rubus ideaus* L. (red raspberries) and *Fragaria* × *ananassa* L. (strawberries) (Miranda-Rottmann *et al.*, 2002; Araya *et al.*, 2006). Céspedes *et al.* (2008) have demonstrated that *A. chilensis* phenolic extracts also showed AC *in vivo* (rat model). The main compounds in *A. chilensis* fruits that have been identified are gentisic acid, ferulic acid, gallic acid, p-coumaric acid, sinapic acid, 4-hydroxybenzoic acid, delphinidin, cyanidin, vanillic acid, delphinidin gallate, gallocatechin gallate, quercetin, rutin, myricetin, catechin and epicatechin, and eight glycosides of anthocyanidins (delphinidin-3-sambubioside-5-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, delphinidin-3-sambubioside, delphinidin-3-glucoside, cyanidin- 3-sambubioside and cyanidin-3-glucoside), and proanthocyanidin B (Escribano-Bailon *et al.*, 2006; Céspedes *et al.*, 2010b).

Fredes (2009) reviewed the current research related to polyphenol identification, AC and polyphenol bioavailability in four native berries (*A. chilensis, Berberis buxifolia, Ugni molinae* and *Fragaria chiloensis*); there is comparatively less research on the antioxidant properties of *B. buxifolia, U. molinae* and *F. chiloensis*.

Ruiz *et al.* (2010) compared polyphenols of *Berberis microphylla* (syn. *B. buxifolia*) fruit with *A. chilensis* and *U. molinae*. Polyphenols in *B. microphylla* were most present in glycosylated form (3-glucoside

conjugates) and 18 anthocyanins were the most abundant: delphinidin-3,5-dihexoside, delphinidin-3-rutinoside-5-glucoside, cyanidin-3,5-dihexoside, petunidin-3,5-dihexoside, petunidin-3-rutinoside-5-glucoside, peonidin-3,5-dihexoside, malvidin-3,5-dihexoside, delphinidin-3-glucoside, delphinidin-3-rutinoside, malvidin-3-rutinoside-5-glucoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, petunidin-3-glucoside, petunidin-3-rutinoside, peonidin-3-glucoside, peonidin-3-rutinoside, malvidin-3-glucoside and malvidin-3-rutinoside. In *U. molinae* fruit two anthocyanins were identified: cyanidin-3-glucoside and peonidin-3-glucoside.

8.4 Polyphenols in Chilean Plants: an Agronomic Perspective

In natural habitats plants are surrounded by an enormous number of potential enemies. Nearly all ecosystems contain a wide variety of bacteria, viruses, fungi, nematodes, mites, insects, mammals and other herbivorous animals. Owing to their sedentary nature, plants cannot avoid these herbivores and pathogens simply by moving away; they must protect themselves in other ways (Taiz and Zeiger, 1991).

PNPs are organic compounds that defend plants against a variety of herbivores and pathogenic microbes. They also may serve as structural support (lignin) or pigments (anthocyanins) (Croteu, 2000). PNPs have no generally recognized, direct roles in primary metabolism (e.g. photosynthesis, respiration and solute transport). They also differ from primary metabolites (amino acids, nucleotides, sugars and acyl lipids) in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or related group of species, whereas primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 1991). From this point of view, all the plants have natural products but the relative contents of these types of compounds in different species are related to various factors that will be described in more detail below.

Flavonoids are derived from cinnamic acid formed by the action of phenylalanine ammonia-lyase enzyme (PAL) starting from the amino acid phenylalanine. PAL acts as the branch point between primary metabolism (shikimic acid metabolic pathway) and secondary metabolism (phenylpropanoid pathway) (Winkel, 2006). Although polyphenols have structural functions in plants, many compounds that are constitutive or tissue specific in a species could be induced in other species or other tissues under stress conditions (Christie *et al.*, 1994). High light/UV, pathogen attack, low nitrogen, low phosphate, low iron, low temperature and wounding are some examples of both abiotic and biotic stress that could induce polyphenols in plants (Dixon and Paiva, 1995).

The main factors that affect polyphenol content in plants may be grouped into genotype, environment, storage and processing, and the stage of harvest (Fig. 8.1).

Genotype refers to the existence of botanical families (e.g. *Labiatae, Rosaceae, Myrtaceae*) with high polyphenol content or a particular type of compound. Within a species there are varieties, selections and ecotypes that have significantly different polyphenol content or have a particular type of such compounds (Fan-Chiang *et al.*, 2005; Scalzo *et al.*, 2005; Lohachoompol *et al.*, 2008; Speisky *et al.*, 2008). The climate, soil and agronomic management affect the polyphenol content in leaves and fruits, and some authors postulate that stressful conditions favour their production (Häkkinen and Törrönen, 2000; Romero *et al.*, 2002; Pedreira dos Santos *et al.*, 2007; Servili *et al.*, 2007; Terry *et al.*, 2007). Storage (fresh and frozen) and agro-industrial processes cause the loss of some types of polyphenols, as well as the transformation of certain compounds (hydrolysable tannins) to monomeric forms that would present a greater bioavailability (Cabrita *et al.*, 2000; Zafrilla *et al.*, 2001; Piljac-Zegarac *et al.*, 2009). In relation to the stage of harvest, leaves and aerial parts of plants collected in different seasons, and fruits at different stages of development and maturity, present different concentrations and

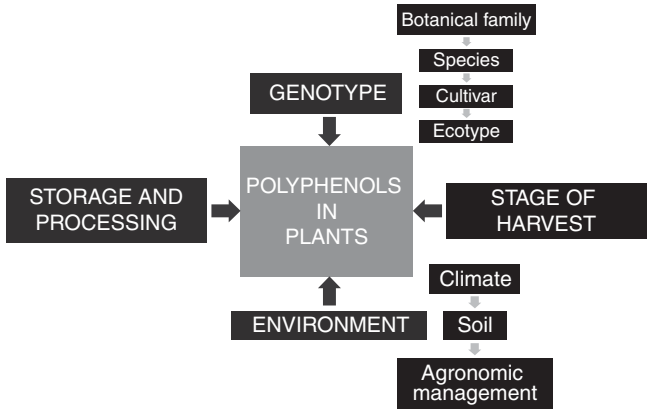

Fig. 8.1. Main factors that affect polyphenol content in plants.

types of polyphenols (Vvedenskaya and Vorsa, 2004; Kulkarni and Aradhya, 2005; Wang *et al.*, 2009).

For these reasons, it is essential to take into account all these factors to optimize the polyphenol content in target plants. Parr and Bolwell (2000) indicated that the modification and/or optimization of the polyphenol content in crops could be made through conventional breeding, specific agronomic practices or through biotechnology. However, the manipulation of the metabolism of polyphenols using molecular techniques could be costly and mean only a small increase in polyphenol content compared to agronomic management. Among the research on Chilean plants, few examples of scientific studies that considered agronomic aspects such as characteristics of ecotypes, environmental conditions where samples were collected, season of harvest for leaves (aerial parts) or maturity stage for fruits exist (Vogel *et al.*, 2002; Rojo *et al.*, 2009; Simirgiotis and Schmeda-Hirschmann, 2010). The main reason that could explain this scenario is that the majority of Chilean medicinal plants are gathered from the wild. Therefore, aspects of their domestic cultivation have not yet been established. Table 8.3 indicates the Chilean plants analysed in this chapter of which some cultivation practices are known.

The cultivation practices in Table 8.3 are related to the treatment of seeds before sowing, vegetative propagation, and climate and soil requirements. This agronomic information, however, is not sufficient. Nevertheless, some efforts in the selection and cultivation of *A. citriodora, A. chilensis, Q. saponaria, B. globosa* and *U. molinae* exist along with some criteria for genetic breeding, propagation, fertilization, irrigation, harvest and postharvest.

Although Chilean plants analysed in this chapter do not have important obstacles to their conservation (except *C. cachanlahuen, L. acaulis* and *L. hirsute*), it is necessary to guarantee their sustainability in the near future if they are to have any potential use in the pharmaceutical and food industries.

8.5 Conclusions

Sufficient scientific evidence supporting the biological activities and antioxidant properties of Chilean plants used as traditional herbal medicine does not exist. Nevertheless, scientific advances related to the identification of polyphenols in some Chilean plants (e.g. *A. chilensis, A. citriodora, B. globosa* and *P. boldus*) have allowed significant validation of their traditional uses. Polyphenol bioavailability is a necessary challenge in terms of identifying the compounds responsible for antioxidant capacity and whether these compounds reach significant plasmatic concentrations.

Table 8.3. Knowledge of Chilean medicinal plant cultivation. (Adapted from INIA, 2010; MINSAL, 2009; Vogel *et al.*, 2002, 2004.)

No knowledge of cultivation practices	Some knowledge of cultivation practices
Acaena splendens	*Aloysia citriodora*
Acantholippia desertícola	*Aristotelia chilensis*
Centaurium cachanlahuen	*Buddleja globosa*
Cestrum parqui	*Calceolaria thyrsiflora*
Chenopodium ambrosioides	*Drimys winteri*
Cuscuta chilensis	*Equisetum bogotense*
Ephedra chilensis	*Fabiana imbricata*
Flaveria bidentis	*Fuchsia magellanica*
Haplopappus spp.	*Geum andicola*
Lampaya medicinalis	*Gunnera tinctoria*
Laretia acaulis	*Libertia sessiliflora*
Luma chequen	*Lomatia hirsuta*
Muehlenbeckia hastulata	*Margyricarpus pinnatus*
Polypodium feuillei	*Maytenus boaria*
Porlieria chilensis	*Otholobium glandulosum*
Senecio fistulosus	*Peumus boldus*
Satureja parvifolia	*Pseudognaphalium viravira*
Tristerix corymbosus	*Quillaja saponaria*
	Quinchamalium chilense
	Salix humboldtiana
	Schinus areira
	Senna stipulacea
	Solanum ligustrinum
	Ugni molinae

On the other hand, there is scientific information of antioxidant properties of Chilean plants that are not included in the lists of traditional herbal medicines. The uses of these Chilean plants have not been significantly promoted to the rest of the country.

Because most of the Chilean medicinal plants are currently gathered in the wild, it is necessary to consider agronomical strategies for their selection, breeding and cultivation based on polyphenol content in order to optimize their antioxidant properties and to guarantee sustainability of these Chilean botanical resources.

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9 Antioxidant Activity of Anacardic Acids

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9.1 Introduction

In recent years the cashew *Anacardium occidentale* L. (Anacardiaceae) apple has increased in value, especially in the countries where it is grown, such as Brazil. There is no doubt that the nut (true fruit) is the most important product of the cashew tree. However, this tree also yields the pear-shaped 'apple' (pseudo fruit) to which the nut is attached. A number of processes have now been developed for converting the cashew apple into various products such as juice, jam, syrup, chutney and beverage. Cashew apple juice is, in fact, one of the most popular juices in Brazil today. In our continuing efforts concerning the utilization of 'cashew nut shell liquid' (CNSL) from *A. occidentale* as a starting material for preparation of useful compounds, anacardic acids and 6-alk(en)ylsalicylic acids were isolated and characterized from the cashew apple and nut. Anacardic acids can be obtained in large quantities from the CNSL (reviewed by Tyman, 1979). Although the CNSL is available in greater tonnage, it is neglected in commercial terms, and there is thus considerable potential for its further exploitation. CNSL is traditionally obtained as a by-product during the process of removing the cashew kernel from the nut. The processes used are mainly hot-oil and roasting in which the CNSL oozes out from the shell and the liquid obtained is about 30–35% from the shell. Subsequently, the diverse biological activities of anacardic acids including antioxidant activity have been described (Masuoka and Kubo, 2004; Ha and Kubo, 2005; Kubo *et al.*, 2006). Antioxidant activity is associated with a lowered incidence of some forms of cancer and cardiovascular diseases (Diplock *et al.*, 1998). Anacardic acids have thus recently also been advocated for use in functional food formulations (Trevisan *et al.*, 2006). These data have, however, been reported as a result of sporadic research, so it is timely to review and synthesize all that is currently known.

The oxidation of unsaturated fatty acids in biological membranes leads to a decrease in the membrane fluidity (Dobrestova *et al.*, 1977) and disruption of membrane structure and function (Machlin and Bendich, 1987; Slater and Cheeseman 1987). Cellular damage owing to lipid peroxidation is associated with carcinogenesis (Yagi, 1987) and other diseases (Garewal, 1997). Inhibition of membrane peroxidation has been shown to have a protective effect in the initiation and

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promotion of certain cancers (Rousseau *et al.*, 1992). The past experimental studies have provided compelling evidence that antioxidants play an important role in reducing the risk of cancer. However, the previous studies usually emphasized the scavenging activity to use as antioxidant additives in food and lack comprehensiveness. The importance of discovering new safe and effective antioxidants is of considerable interest in preventive medicine. Antioxidants isolated from regularly consumed foods and beverages, such as the cashew apple and its processed products, may be superior to non-natural products. Therefore, our investigation has been further extended to test the antioxidation activity of anacardic acids. Because anacardic acids are the derivatives of salicylic acid (Machlin and Bendich, 1987) with a nonisoprenoid alk(en)yl side chain, their activity is compared with that of salicylic acid.

9.2 Experimental

9.2.1 Chemicals

Anacardic acids (**1**–**3**) and the corresponding cardanols (**4**–**6**) used for the assay were previously isolated from the cashew nut shell oil (Fig. 9.1). Their re-purification by recycle high-performance liquid chromatography (R-HPLC) was achieved using an ODS C_{18} column (Kubo *et al.*, 1986). Salicylic acid, linoleic acid, BHT, EDTA, thiobarbituric acid (TBA), 1,1-diphenyl-2-*p*-picrylhydrazyl (DPPH), 2,2´-azo-*bis*(2-amidinopropane) dihydrochloride (AAPH), ADP, bovine serum albumin and nitroblue tetrazolium were purchased from Sigma Chemical Co. (St. Louis, MO).

9.2.2 Assay of autoxidation

Oxidation of linoleic acid was measured by the modified method described previously (Haraguchi *et al.*, 1992). Different amounts of samples dissolved in 30 µl ethanol were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 0.57 ml of 2.51% linoleic acid in ethanol and 2.25 ml of 40 mM phosphate buffer (pH 7.0). The vial was placed in an oven at 40°C. After 5 days incubation a 0.1 ml aliquot of the mixture was diluted with 9.7 ml of 75% ethanol, which was followed by adding 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture the absorbance at 500 nm was measured.

9.2.3 Radical scavenging activity on DPPH

First, 1 ml of 100 mM acetate buffer (pH 5.5), 1.87 ml of ethanol and 0.1 mL of ethanolic solution of 3 mM of DPPH were put into a test tube. Then, 0.03 ml of the sample solution (dissolved in DMSO) was added to the test tube and incubated at 25°C for 20 min. The absorbance at 517 nm (DPPH, $\varepsilon = 8.32 \times 10^3\ M^{-1}cm^{-1}$) was recorded. As control, 0.03 ml of DMSO was added to the test tube. From the decrease in absorbance, scavenging activity was calculated and expressed as scavenged DPPH molecules per one molecule.

9.2.4 Assay of superoxide anion generated by xanthine oxidase

The xanthine oxidase (EC 1.1.3.22, Grade IV) used for the bioassay was purchased from Sigma Chemical Co. Superoxide anion was generated enzymatically by the xanthine oxidase system. The reaction mixture consisted of 2.70 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine, 0.03 ml of 0.5 % bovine serum albumin, 0.03 ml of 2.5 mM nitroblue tetrazolium and 0.06 ml of sample solution (dissolved in DMSO). To the mixture at 25°C, 0.12 ml of xanthine oxidase (0.04 units) was added, and the absorbance at 560 nm was recorded for 60 s (by formation of blue formazan) (Toda *et al.*, 1991). The control experiment was carried

out by replacing the sample solution with the same amount of DMSO.

9.2.5 Assay of uric acid generated by xanthine oxidase

The reaction mixture consisted of 2.76 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine and 0.06 ml of sample solution (dissolved in DMSO). The reaction was started by the addition of 0.12 ml of xanthine oxidase (0.04 units), and the absorbance at 293 nm was recorded for 60 s.

9.2.6 Lipoxygenase assay

The soybean lipoxygenase-1 (EC 1.13.11.12, Type 1) used for the bioassay was purchased from Sigma Chemical Co. Throughout the experiment linoleic acid was used as a substrate. In the current spectrophotometric experiment, the enzyme activity of soybean lipoxygenase-1 monitored at 25°C by Spectra MAX plus spectrophotometer (Molecular Device, Sunnyvale, CA). The enzyme assay was performed as previously reported (Kemal *et al.*, 1987) with slight modification. In general, 5 µl of an ethanolic inhibitor solution was mixed with 15 µl of 3 mM stock solution of linoleic acid and 2.97 ml of 0.1 M sodium borate buffer (pH 9.0) in a quartz cuvette. Then, 10 µl of 0.1 M sodium borate buffer solution (pH 9.0) of lipoxygenase (0.52 µM) was added. The resultant solution was mixed well and the linear increase of absorbance at 234 nm, which expressed the formation of conjugated diene hydroperoxide (13-HPOD, $\varepsilon = 2.50 \times 10^4\ M^{-1}\ cm^{-1}$), was measured continuously. The lag period shown on lipoxygenase reaction (Ruddat *et al.*, 2003) was excluded for the determination of initial rates. The stock solution of linoleic acid was prepared with Tween-20 and sodium borate buffer at pH 9.0, and then total Tween-20 content in the final assay was adjusted below 0.002%. For pre-incubation experiments the enzyme was incubated with various concentrations of compounds in 0.1 M sodium borate buffer (pH 9.0) at 25°C. At timed intervals, reactions were started by adding 15 µM linoleic acid.

9.2.7 Data analysis and curve fitting

The assay was conducted in triplicate with separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc, Chicago, IL). The IC_{50} values were obtained by fitting experimental data to the logistic curve by Langmuir isotherm as follows (Copeland, 2000):

$$\text{Activity (\%)} = 100(1/(1+([I]/IC_{50}))$$

Inhibition mode was analysed with Enzyme Kinetics Module 1.0 (SPSS Inc) equipped with Sigma Plot 2000.

9.3 Anacardic Acids

Three of the major anacardic acids isolated from the *A. occidentale* apple are: 6[8´(*Z*), 11´(*Z*),14´-pentadecatrienyl]salicylic acid (**1**), 6[8´(*Z*),11´(*Z*)-pentadecadienyl]salicylic acid (**2**) and 6[8´(*Z*)-pentadecenyl]salicylic acid (**3**). They are referred to for simplicity as anacardic acid ($C_{15:3}$), anacardic acid ($C_{15:2}$) and anacardic acid ($C_{15:1}$), respectively (Fig. 9.1). Their fully saturated derivative analogue, 6-pentadecylsalicylic acid (**4**), referred to as anacardic acid ($C_{15:0}$), was not isolated from *A. occidentale* but rather isolated as a prostaglandin synthetase inhibitor from an African medicinal plant *Oziroa mucronata* (Anacardiaceae) together with anacardic acid ($C_{15:1}$) (Kubo *et al.*, 1987). Because anacardic acids are the derivatives of salicylic acid (Machlin and Bendich, 1987) with a nonisoprenoid alk(en)yl side chain, their activity was compared with that of salicylic acid (**5**). The availability of cardanol, 3[8´(*Z*), 11´(*Z*), 14´-pentadecatrienyl]phenol, referred to as cardanol ($C_{15:3}$) (**6**), an artefact of the corresponding anacardic acid ($C_{15:3}$) obtained by heating treatment from the same source, is an additional benefit for comparison. Anacardic acid ($C_{15:3}$) was selected for the present study as

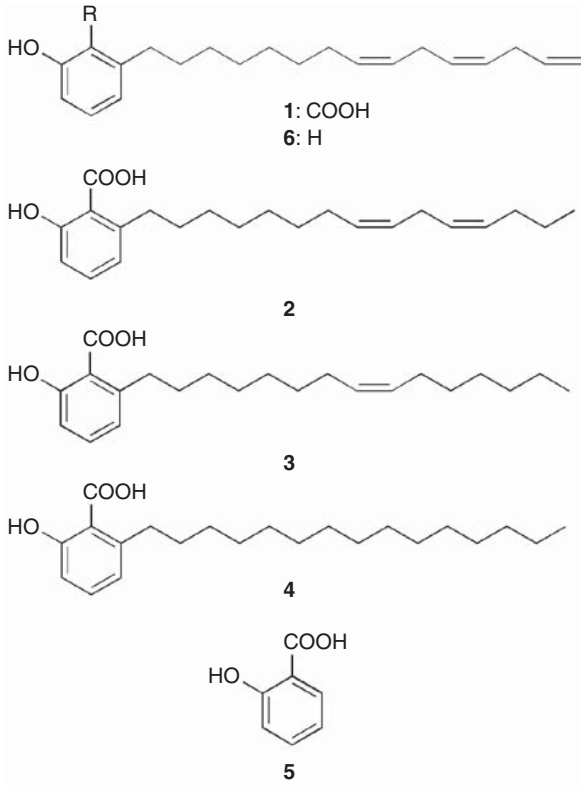

Fig. 9.1. Chemical structures of anacardic acids and related compounds. See text for names.

a model, because this particular anacardic acid was available from our previous study (Kubo *et al.*, 1986).

9.4 Lipid Peroxidation

Lipid peroxidation is known to be one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. Membrane lipids are abundant in unsaturated fatty acids. Linoleic acid is particularly a target of lipid peroxidation. The effect of anacardic acid ($C_{15:3}$) and salicylic acid on the autoxidation of linoleic acid was first tested by the ferric thiocyanate method as previously described (Osawa and Namiki, 1981). In a control reaction, the production of lipid peroxide increased almost linearly during 8 days of incubation. α-Tocopherol, also known as vitamin E, inhibited linoleic acid peroxidation by almost 50% at a concentration of 30 μg/ml. However, neither anacardic acid ($C_{15:3}$) nor salicylic acid inhibited this oxidation at the same concentration. The negative result of anacardic acid ($C_{15:3}$) can be explained by their structural feature in which the electron-donating alkenyl group is located at the *meta*-position to the hydroxyl group so that it does not stabilize the phenoxy radicals (Cuvelier *et al.*, 1992). Salicylic acid does not possess any alkyl group, however. Cardanol ($C_{15:3}$) inhibited linoleic acid peroxidation by about 30% at a concentration of 30 μg/ml, but this inhibitory activity is still less than that of α-tocopherol. The results observed indicate that anacardic acids are unlikely to act as radical scavengers because they do not have the ability to donate a hydrogen atom to the peroxy radical derived from the auto-oxidizing fatty acids. Further evidence for this conclusion

was obtained by a more direct experiment for the radical scavenging activity that can be measured as decolorizing activity following the trapping of the unpaired electron of DPPH. None of anacardic acids (**1**–**3**) exhibited notable radical scavenging activity (0.01 ± 0.02 scavenged DPPH molecule per anacardic acid molecule). On the basis of the above results, further study was initiated.

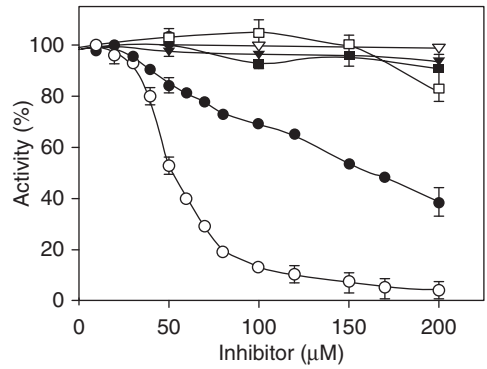

Fig. 9.2. Inhibition of superoxide anion and uric acid by xanthine oxidase with anacardic acid ($C_{15:3}$) and salicylic acid. Reaction rates by xanthine oxidase were measured at 200 μM xanthine in the presence of 0–200 μM anacardic acid, cardanol and salicylic acid. ○, Superoxide anion generation rates and ●, uric acid generation rates in the presence of anacardic acid ($C_{15:3}$). △, Superoxide anion generation rates and ▲, uric acid generation rates in the presence of salicylic acid. □, Superoxide anion generation rates and ■, uric acid generation rates in the presence of cardanol.

9.5 Xanthine Oxidase

The human body is known to produce free radicals during the course of its normal metabolism. Free radicals are even required for several normal biochemical processes. For example, the phagocyte cells involved in the body's natural immune defences generate free radicals in the process of destroying microbial pathogens. If free radicals are produced during the normal cellular metabolism in sufficient amounts to overcome the normally efficient protective mechanisms, metabolic and cellular disturbances will occur in a variety of ways. Evidence is accumulating that extracellular free radicals are also produced *in vivo* by several oxidative enzymes in the human body other than phagocytes. For example, xanthine oxidase (EC 1.1.3.22), a molybdenum-containing enzyme, produces the superoxide anion ($O_2^{\bullet -}$) radical as a normal product (Fong *et al.*, 1973). The one-electron reduction products of O_2, the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxy radical ($HO^{\bullet}$) from $O_2^{\bullet -}$, participate in the initiation of lipid peroxidation (Comporti, 1993). Superoxide is also produced during mitochondrial respiration (Halliwell and Gutteridge, 1990a) and by NADPH oxidase (Pagano *et al.*, 1995), cyclooxygenase and lipoxygenase (Kukreja *et al.*, 1986), nitric oxidase synthetase (NOS) (Cosentino *et al.*, 1998) and cytochtome P450 (Fleming *et al.*, 2001). The effect of anacardic acids on the generation of the superoxide anion by xanthine oxidase was tested and the result is shown in Fig. 9.2. In the control, the superoxide anion generated by the enzyme reduces yellow nitroblue tetrazolium to blue formazan. Hence, the superoxide anion can be detected by measuring the absorbance of formazan produced at 560 nm. At a concentration of 30 μg/ml, anacardic acid ($C_{15:3}$) (88 μM) inhibited this formazan formation 82 ± 4%. Interestingly, salicylic acid did not show any observable inhibitory activity up to a concentration of 138 μg/ml (1.0 mM) and showed 7 ± 3% inhibition at 276 μg/ml, indicating that the C_{15}-alkenyl side chain is associated with this inhibitory activity. Cardanol did not show this inhibitory activity up to 0.2 mM, indicating that the structure of 2-carboxylphenol (salicylic acid) is also necessary. As the concentrations of anacardic acid ($C_{15:3}$) increased, the remaining enzyme activity was rapidly decreased. Notably, this inhibition mechanism does not follow hyperbolic inhibition by anacardic acid concentration (Michaelis–Menten equation) but follows the Hill equation (Beckmann *et al.*, 1998) instead. The shape of the inhibition curve of xanthine oxidase by anacardic acid ($C_{15:3}$) is sigmoidal (*S*-shaped) (IC_{50} = 51.3 ± 1.5 μM) as shown in Fig. 9.3. This inhibition occurred over a

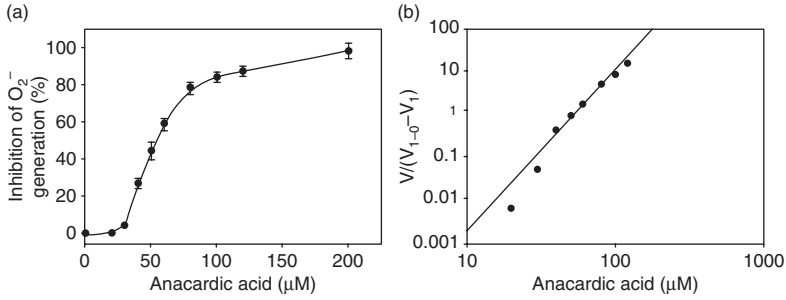

Fig. 9.3. Inhibited rates of the superoxide anion generation by anacardic acid ($C_{15:3}$) and the Hill plot analysis. (a) Inhibited rates of superoxide anion generation were calculated from those of superoxide anion generation by xanthine oxidase in the presence of 0–200 μM anacardic acid ($C_{15:3}$) at 200 μM xanthine. (b) The rates were plotted according to the Hill equation.

very narrow range of anacardic acid ($C_{15:3}$) concentration (0.04–0.14 mM), which is much less than a usual simple equilibrium that would occur over a 100-fold concentration range. This indicates only tight binding of inhibitor, but the curve of inhibition rate followed a Hill equation with a slope factor of 4.2 ± 0.5. This suggests that anacardic acid ($C_{15:3}$) binds cooperatively to xanthine oxidase (Bray, 1963). It should be noted, however, that a common naturally occurring antioxidant, α-tocopherol, is less effective in scavenging the superoxide anion generated by the xanthine oxidase and the IC_{50} is 220 ± 20 μM (Masuoka and Kubo, 2004).

It seems that the antioxidant activity of anacardic acids is not due to radical scavenging but to inhibiting the enzyme activity. In order to verify this conclusion the formation of uric acid was measured, because xanthine oxidase is known to convert xanthine to uric acid. This enzyme-catalysed reaction proceeds via the transfer of an oxygen atom to xanthine from the molybdenum centre. The inhibition mechanism also does not follow hyperbolic inhibition by anacardic acid concentration (Fig. 9.2) but follows the Hill equation instead. The shape of the inhibition curve of xanthine oxidase by anacardic acid ($C_{15:3}$) is sigmoidal (IC_{50} = 162 ± 10 μM). The curve of inhibition rate followed a Hill equation with a slope factor of 1.7 ± 0.2. This result confirmed that anacardic acid ($C_{15:3}$) binds by cooperative binding to xanthine oxidase and affects the uric acid formation less than the superoxide anion formation. Interestingly, salicylic acid did not inhibit the enzyme up to 200 μM (27.6 μg/ml) but cooperatively inhibited at higher concentration (IC_{50} = 580 ± 28 μM). The result obtained indicates that the alkyl side chain plays an important role in eliciting the activity. The hydrophobic interaction alone is not enough, however, to elicit the xanthine oxidase inhibitory activity because cardanol ($C_{15:3}$), which possesses the same side chain as anacardic acid ($C_{15:3}$), did not exhibit any inhibitory activity.

9.6 Lipoxygenase

Lipoxygenase (EC 1.13.11.12) is a non-haem iron enzyme that catalyses the dioxygenation of polyunsaturated fatty acids containing a 1(*Z*),4(*Z*)-pentadiene system, such as linoleic acid and arachidonic acid, into their 1-hydroperoxy-2(*E*),4(*Z*)-pentadiene product (Shibata and Axelrod, 1995). Lipoxygenases are therefore of importance because they could generate peroxides in human low-density lipoproteins (LDLs) *in vivo* and facilitate the development of arteriosclerosis, a process in which lipid peroxidation seems to be intimately involved (Cornicelli and Triredi, 1999; Kris-Etherton and Keen, 2002). Lipid peroxidation is a typical free-radical oxidation and proceeds via a cyclic chain reaction (Witting, 1980).

It is also well known, however, that lipid peroxidation is one of the major factors in deterioration during the storage and processing of foods, because it can lead to the development of unpleasant rancid or off flavours as well as potentially toxic end products. In our preliminarily assay we became aware that anacardic acid ($C_{15:3}$) and anacardic acid ($C_{15:2}$) were oxidized as substrates at lower concentrations (<40 μM) because both possess a 1(*Z*),4(*Z*)-pentadiene system in their C_{15}-alkenyl side chain. Hence, the inhibition kinetics were emphasized with anacardic acid ($C_{15:1}$), although both anacardic acid ($C_{15:3}$) and anacardic acid ($C_{15:2}$) inhibited the oxidation of linoleic acid catalysed by soybean lipoxygenase-1 (EC 1.13.11.12, Type 1) at a higher concentration (>40 μM).

The oxidation of linoleic acid catalysed by soybean lipoxygenase-1 follows Michaelis–Menten kinetics. The kinetic parameters for this oxidase obtained from a Dixon plot show that the K_m value is 11.7 μM and V_m is 4.8 μM/min. The estimated value of K_m obtained using a spectrophotometric method is in good agreement with the previously reported value (Schilstra *et al.*, 1992; Berry *et al.*, 1997). The kinetic and inhibition constants obtained are listed in Table 9.1. As illustrated in Fig. 9.4, the inhibition kinetics analysed by Dixon plots show that anacardic acid ($C_{15:1}$) is a competitive inhibitor because increasing anacardic acid ($C_{15:1}$) resulted in a family of lines with a common intercept on the $1/v$ axis but with different slopes. The equilibrium constant for inhibitor binding, K_I, was obtained from a plot of the apparent Michaelis–Menten constant versus the concentration of anacardic acid ($C_{15:1}$), which is a linear. The inhibition kinetics analysed by Lineweaver–Burk plots also confirmed that the anacardic acid ($C_{15:1}$) is a competitive inhibitor (data not shown). A similar result was also obtained by monitoring oxygen consumption and the results are listed in Table 9.1. The estimated value

Table 9.1. Kinetics and inhibition constants of anacardic acid ($C_{15:1}$) for soybean lipoxygenase-1.

	Inhibition	
	Increase of A_{234}	O_2 consumption
IC_{50}	6.8 μM	31.5 μM
K_m	11.7 μM	43 μM
V_m	4.8 μmol/min	6.5 μmol/min
Inhibition	Reversible	Reversible
Inhibition type	Competitive	Competitive
K_I	2.8 μM	14.2 μM

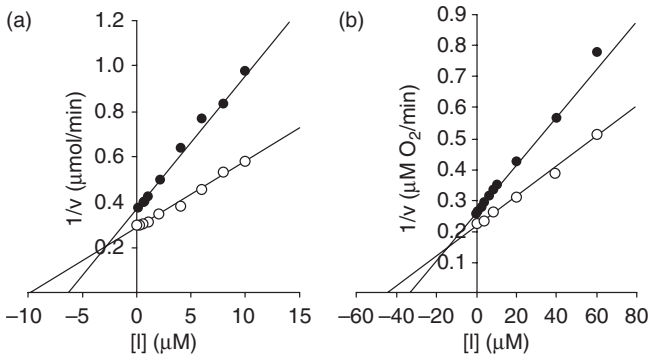

Fig. 9.4. Dixon plots of 13-HPOD generation and oxygen consumption by soybean lipoxygenase-1 in the presence of anacardic acid ($C_{15:1}$) in borate buffer (pH 9.0) at 25°C. (a) Plots of 13-HPOD generation (increase of A234 nm). Concentrations of linoleic acid substrate for curves • and ○ were 15 and 30 μM, respectively. K_m is equal to 11.7 μM, K_I is equal to 2.8 μM and V_m is equal to 4.8 μmol/min. (b) Plots of oxygen consumption. Concentrations of linoleic acid for curves were 50 (•) and 80 (○) μM, respectively. K_m is equal to 43 μM, K_I is equal to 14.2 μM and V_m is equal to 6.5 μmol/min.

of K_m is approximately fourfold higher than that obtained using a spectrophotometric method. This is in good agreement with the previously reported observations (Berry *et al.*, 1997).

Salicylic acid (Machlin and Bendich, 1987) did not inhibit soybean lipoxygenase-1 up to 200 μM, suggesting that a pentadecenyl side chain is essential to elicit the activity. The pentadecenyl side chain alone is not enough, however, to elicit the activity because cardanol ($C_{15:1}$), which possesses the same side chain as anacardic acid ($C_{15:1}$), acted neither as a substrate nor as an inhibitor.

As far as the present cell-free experiment using soybean lipoxygenase-1 is concerned, the inhibition kinetics observed do not exceed 5 min; however, much longer observation is needed from a practical point of view. The time course of oxidation of linoleic acid catalysed by soybean lipoxygenase-1 in the presence of different anacardic acid ($C_{15:1}$) concentrations is shown in Fig. 9.5. At each concentration of anacardic acid ($C_{15:1}$) the rate slowly decreased with increasing time until a straight line ran parallel to the x-axis, indicating that the enzyme activity was lost.

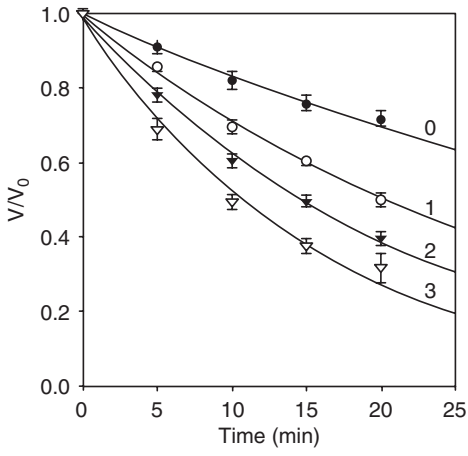

Fig. 9.5. Time dependence of the fractional velocities for the catalysis of linoleic acid soybean lipoxygenase-1 in the presence of several concentrations of anacardic acid ($C_{15:1}$). Conditions were: 0.1M sodium borate buffer, pH 9.0, linoleic acid 30 μM and 0.188 μg/ml soybean lipoxygenase-1. Concentrations of anacardic acid ($C_{15:1}$) for curves 0–3 were 0.8, 2, 4 and 6 μM, respectively.

9.7 Conclusion

The oxidative degradation of polyunsaturated fatty acids occurs in two sequential steps of initiation and propagation (Svingenn *et al.*, 1979). Antioxidative materials acting in living systems are therefore classified as preventive antioxidants and chain-breaking ones (Halliwell and Gutteridge, 1990b). In view of the present investigation, it seems that the antioxidant activity of anacardic acids is not due to radical scavenging but to preventing. They may be advantageous to suppress the formation of free radicals and active oxygen species in the first line of defence. Safety is a primary consideration for antioxidants in food products. In connection with this, the radical-scavenging antioxidant traps an active radical and the antioxidant-derived radical is formed. The fate of this newly formed radical is important in determining the total potency of the antioxidant. For example, several inhibitors of lipid peroxidation have the potential to accelerate free-radical damage to other biomolecules (Halliwell *et al.*, 1995). Because of this Janus-like property, scavenging antioxidants are also known as a double-edged sword. The data so far obtained indicate the advantage of anacardic acids as preventive antioxidants. In addition, the fact that anacardic acids are known in the cashew apple and nut, which have been continuously consumed by people for many years, should give them another considerable advantage as antioxidants.

Anacardic acids were previously reported to have high selectivity toward transition metal ions, especially Fe^{2+} and Cu^{2+} (Nagabhushana *et al.*, 1995). The ability of the high selectivity of chelation toward Fe^{2+} and Cu^{2+} of anacardic acids should give them considerable advantage

as antioxidants (Arora *et al.*, 1998). These transition metal ions are well known as powerful promoters of free-radical damage in both the human body (Halliwell and Gutteridge, 1989; Henel and Linn, 1997) and foods (Aruoma and Halliwell, 1991). For example, anacardic acids may prevent cell damage induced by H_2O_2 because this can be converted to the more reactive oxygen species, hydroxy radicals, in the presence of these metal ions (Lodovici *et al.*, 2001). Salicylic acid does not have this high selectivity of chelation, so the alk(en)yl side chain in anacardic acids is also related to the high selectivity towards transition metal ions. It seems that anacardic acids act as antioxidants in a variety ways, including inhibition of various prooxidant enzymes involved in the production of the reactive oxygen species and chelate divalent metal ions such as Fe^{2+} or Cu^{2+}, but do not quench reactive oxygen species.

An antioxidant is, as a general definition, any substance capable of preventing oxidation. Deleterious free-radical-mediated oxidations occur in aerobic organisms as a result of normal oxygen metabolism. Iron, especially ferrous iron (Fe^{2+}), is able to trigger oxidations by reducing as well as by decomposing previously formed peroxides. Hence, an antioxidant that protects from iron toxicity is a substance that can: (i) chelate ferrous iron and prevent the reaction with oxygen or peroxides; (ii) chelate iron and maintain it in a redox state that makes iron unable to reduce molecular oxygen; and (iii) trap already formed radicals, which is a putative action of any substance that can scavenge free radicals in biological systems, regardless of whether they originate from iron-dependent reactions or not (Fraga and Oteiza, 2002).

The preventive antioxidant activity of anacardic acids largely comes from their ability to inhibit various oxidative enzymes. It should be noted, however, that these oxidases produce free radicals in the human body as normal products. Hence, anacardic acids or their metabolites need to reach the sites where the enzymes are located in living systems and need to regulate the enzyme activity to prevent the generation of only unnecessary radicals. For instance, xanthine oxidase occurs almost exclusively in the liver and small intestinal mucosa in mammals. It is not clear if anacardic acids or their metabolites can reach the site and regulate this cellular enzyme activity. If anacardic acids act as highly effective xanthine oxidase inhibitors in the human body, they can be toxic because this oxidase is a normal enzyme involved in purine metabolism. Paradoxically, xanthine oxidase inhibitors are useful to treat some diseases such as gout and urate calculus by regulating uric acid formation. In any case, it seems that anacardic acids have antioxidant activity as a result of inhibiting oxidation-related enzymes and these 6-alk(en)ylsalicylic acids are contained in quantities in the cashew nut and apple. Their role as antioxidants in the human body is unknown, however, when orally ingested, but there are several possibilities. The ingested anacardic acids are: (i) absorbed into the system through the intestinal tract and delivered to the places where antioxidants are needed, preventing the generation of unnecessary radicals; (ii) absorbed but metabolized to inactive forms or are not delivered to the right places; or (iii) not absorbed and excreted. The relevance of the *in vitro* experiments in simplified systems to *in vivo* protection from oxidative damage should be carefully considered. The results obtained indicate that further evaluation is needed from not only one aspect but from a whole and dynamic perspective.

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10 Alkylresorcinols: Chemical Properties, Methods of Analysis and Potential Uses in Food, Industry and Plant Protection

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10.1 Introduction

Phenolic compounds represent a large group of molecules widely distributed in the plant kingdom, where they have a variety of functions in growth, development and defence. These natural products include signalling molecules, pigments and flavours that can attract or repel, as well as allelochemicals that can protect plants against insects, fungi, bacteria and viruses (Posmyk and Janas, 2009). Phenolic lipids or long-chain phenols belong to this type of molecule and are of increasing interest in phytochemistry. This chapter focuses on the non-isoprenoid alkyl derivatives of resorcinol, also known as alkylresorcinols (ARs). Until now, several good reviews were dedicated to different aspects of ARs (Kozubek and Tyman, 1999, 2005; Kozubek *et al*., 2001; Zarnowski and Kozubek, 2002; Ross *et al*., 2004a,c). Here, we revise ARs in the light of our own research interests, including plant sources, methods of analysis, biological role in plants and microorganisms, chemical properties, bioactivities and their possible uses in nutrition, agriculture and industry.

10.2 Chemical Properties and Biosynthesis

The chemical structure of 5-n-ARs is characterized by a benzene ring with two hydroxyl groups located at positions 1 and 3 (the hydrophilic head), and a non-isoprenoid alkyl chain attached at position 5 (the hydrophobic tail; Fig. 10.1). Orcinol is the simplest homologue of the 1,3-dihydroxy-5-n-alk(en)ylbenzene series. Their homologues differ in the alkyl tail and are classified according to a combination of tail length (11–29 carbons) and the degree of unsaturation (0–4 double bonds). These compounds have a strong amphiphilic character that is a consequence of the hydrophilic and hydrophobic regions previously indicated. This characteristic is an important aspect with regard to AR analysis, absorption, metabolism and potential bioactivity. The ARs are

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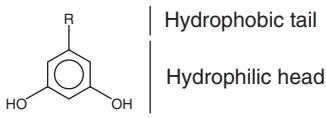


Fig. 10.1. The general structure of alkylresorcinols commonly found in cereal grains. R is a linear alkyl chain that can be saturated, unsaturated and/or have different degrees of oxygenation.

practically insoluble in water and have very low critical micelle concentrations, in the range of 4.5–8.5 mM, varying according to the tail length and degree of unsaturation of the homologue considered. The hydrophobicity of these compounds is also reflected in their high values of octanol/water partition coefficients, which explain their easy incorporation into the phospholipid bilayers. Plants are able to increase the polarity of these substances by glycosylation as suggested by the discovery in recent years of 5-n-AR glycosides in the leaves of *Grevillea robusta* and root exudates from rice seedlings (Kong *et al.*, 2002; Yamashita *et al.*, 2008). Nevertheless, most 5-n-ARs discovered until today were found as aglycones, especially in cereal seedlings and grains (Zarnowsky and Kozubek, 2002).

The biosynthesis of ARs, and phenolic lipids in general, was predicted through the polyketide pathway (Vickery and Vickery, 1981). In this interpretation, biosynthesis starts with a fatty acid unit commonly known as the acyl-CoA starter unit (Route A, Fig. 10.2). A polyketide synthase catalyses the condensation of 3 malonyl-CoA units to the fatty acyl-CoA starter unit, followed by second and third extensions of malonyl-CoAs to 3-oxoacyl-acyl carrier proteins (3-oxoacyl-ACPs), yielding a polyoxomethylene intermediate **2** in which all the carbonyl oxygens are retained. An aldol condensation of the acyclic intermediate **2** and the reduction of the keto groups to hydroxyl groups yields the intermediate **3a**. The release of ACP and decarboxilation of **3a** yields a 5-AR **4** with an odd-carbon-number chain. The liberation of ACP from **3a** affords a 6-alkylresorcinolic acid **5**. This biosynthetic pathway was suggested for short-chain ARs and their reduced analogues, based on labelling experiments using malonate and acetate where the acetyl-CoA is the starter unit. No labelled acetate was detected into the side-chain moiety of longer chain ARs, however, suggesting that in such cases a convergence of the fatty acid and polyketide pathways occurs (Fate and Lynn, 1996). In agreement with this explanation, further research showed that fatty acid units act as direct precursors to form the side-chain moiety of alkylresorcinols (Suzuki *et al.*, 2003). In this case, the intermediate **3a** is orcellinic acid-ACP (R = H), which seems to condense onto a pre-existing fatty acid unit **1b**, affording a 6-(2′-oxoalkyl)-resorcinolic acid **7**, as shown in Route B of Fig. 10.2. The reduction of the 2′-oxo group to a methylene group affords 6-alkylresorcinolic acid **3b**, which can be decarboxylated to liberate 5-AR.

Research on the biosynthethic pathway of sorgoleone, an allelopathic quinone synthesized and exuded from root hairs of *Sorghum bicolor*, showed that the lipid tail and the phenol head of the intermediate 5-pentadecatriene resorcinol are synthesized in different subcellular compartments (Dayan *et al.*, 2003). The 16:3 fatty acid precursor of the tail is synthesized in the plastids by the combined action of fatty acid synthase and desaturases. Then, it is exported out of the plastids and converted to 5-pentadecatriene resorcinol by a type III polyketide synthase similar to a stilbene synthase, because it catalyses the C2 to C7 aldol intramolecular cyclization resulting in orsellinic acid-type rings. In rye seedlings relative high levels of ARs were found in plastids and mitochondria, suggesting that these phenolic lipids may be synthesized in such compartments (Deszcz and Kozubek, 2000).

10.3 Sources of Alkylresorcinols

The ARs have been found in higher plants, algae, mosses, fungi and bacteria. In plants they were detected for the first time in *Ginkgo biloba*, a gymnosperm tree (Morimoto *et al.*, 1968). Further research showed the presence of ARs in angiosperm species, including

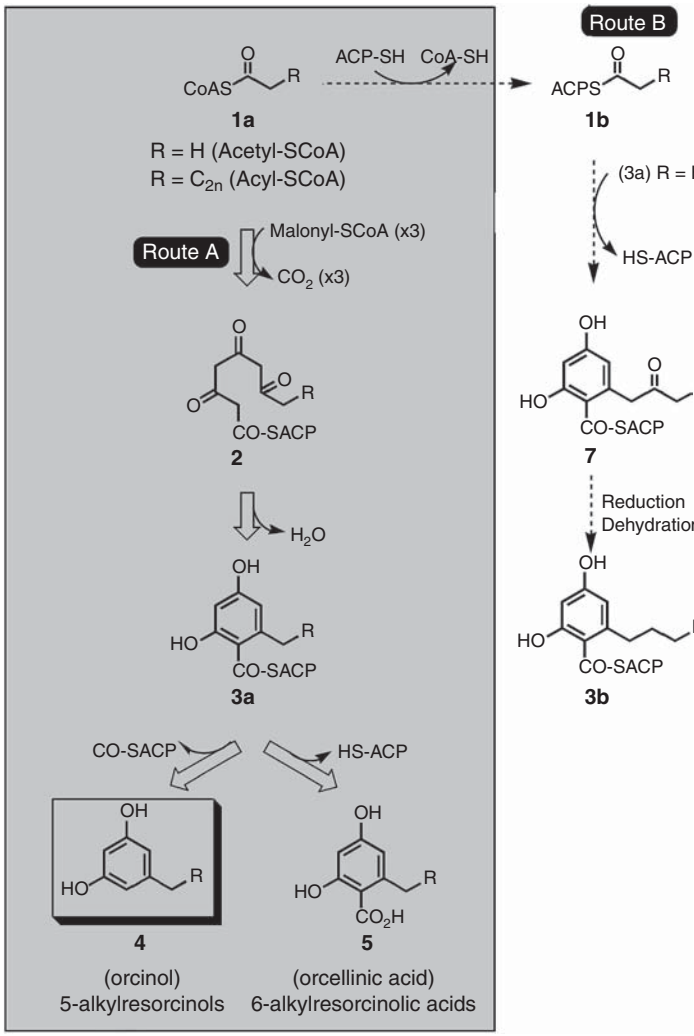
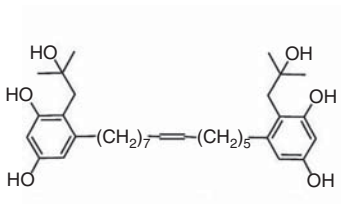
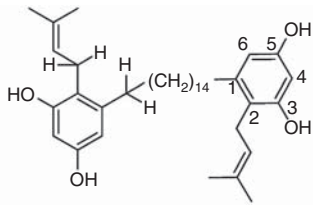


Fig. 10.2. Hypothetical pathways involved in the biosynthesis of alkylresorcinols (ARs).

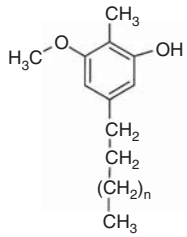
Anacardiaceae, Gramineae, Proteaceae, Myrsinaceae, Primulaceae, Myristinaceae, Iridaceae, Araceae, Asteraceae and Fabaceae (Kozubek and Tyman, 1999; Kozubek *et al.*, 2001; Zarnowsky and Kozubek, 2002). Although most ARs occur as mixtures containing several homologues differing in the length of the alkyl chain and degree of unsaturation, several plants also produce AR derivatives with modifications in the ring, the alkyl side chain or both (Fig. 10.3). Some examples are: the prenyl bis-resorcinols from stems of *Grevillea floribunda* and bis-resorcinols from leaves of *Oncostemon bojerianum*, which contain two benzene rings attached to both ends of the alkyl or alkenyl chain (Prakash Chaturvedula *et al.*, 2002; Wang *et al.*, 2009); cichoriols A–D, isolated from aerial parts of *Cichorium spinosum*, which are 5-n-ARs with a methoxy group and a methyl group attached at positions 3 and 2 of the benzene ring, respectively (Melliou *et al.*, 2003); 5-n-AR derivatives from *Ononis* with free or modified hydroxy and/or keto substituents in the alkyl chain and of the ring-attached hydroxylic groups to form



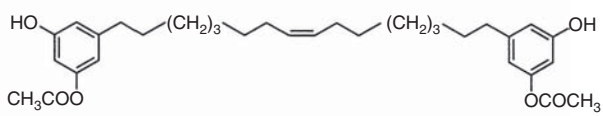
grebustol B
(leaves of *Grevillea robusta*)



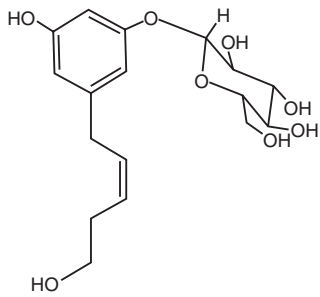
bisnorstriatol
(stems of *Grevillea floribunda*)



1: n = 16 cichoriol A
2: n = 18 cichoriol B
3: n = 19 cichoriol C
4: n = 20 cichoriol D
(aerial parts of *Cichorium spinosum*)



oncostemonol A



grevilloside A
(leaves of *Grevillea robusta*)

Fig. 10.3. Alkylresorcinol derivatives isolated from plant sources.

l,3-dihydroxy-2-alkyl and l,3-dihydroxy-2,5-dialkylbenzenes (Barrero *et al.*, 1994); grevillosides isolated from leaves of *Grevillea robusta*, which are 5-n-ARs O-glucosylated at position 1 of the benzene ring (Yamashita *et al.*, 2008); and the 5-n-AR glycosides of arabinose found in root exudates of rice seedlings (Kong *et al.*, 2002).

The Anacardiaceae and Gramineae are important sources of ARs. In Anacardiaceae, the cashew tree (*Anacardium occidentale*), originating from Brazil, is the major and the cheapest source of meta alkylphenols available in Latin America (De Lima *et al.*, 2008). The fruit of the tree consists of an outer shell (epicarp), a tight-fitting inner shell (endocarp), and a strongly vesicant cashew nut-shell liquid (CNSL, Fig. 10.4a) contained between the inner and outer shell (pericarp). This oily liquid is extracted by roasting cashew nuts. On the basis of the mode of extraction, CNSL is classified into

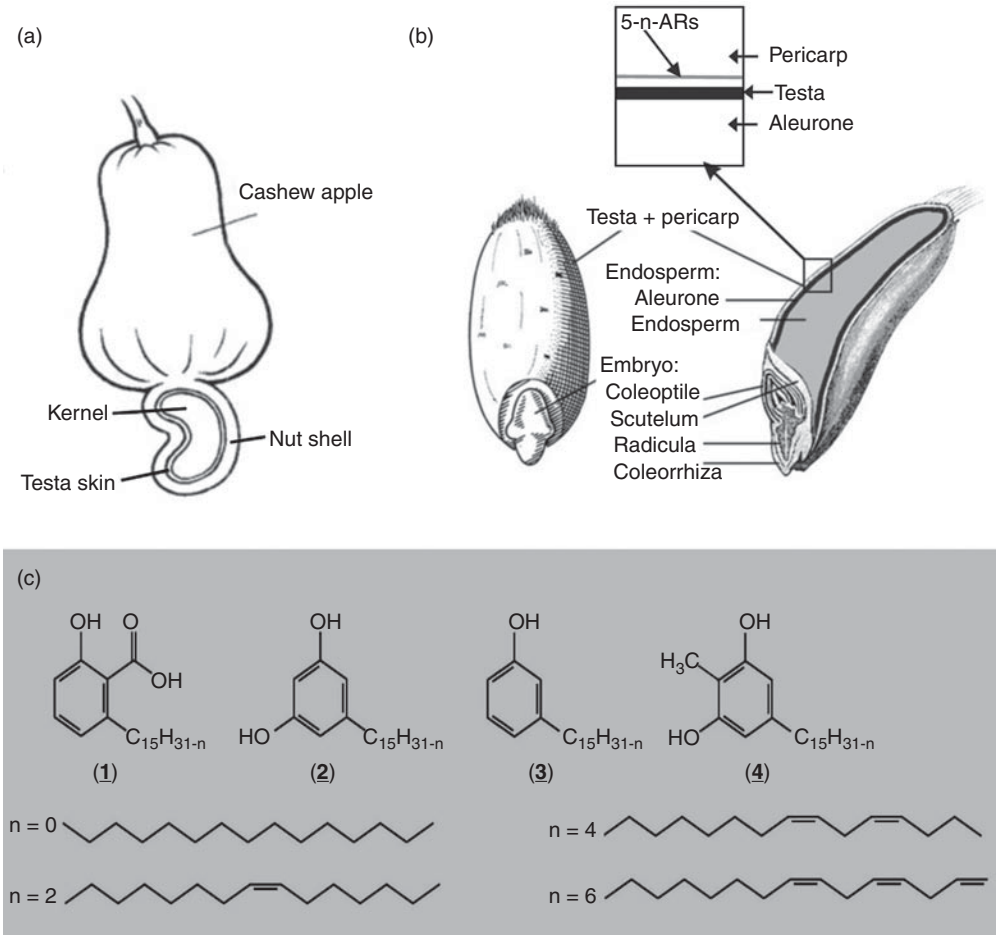


Fig. 10.4. Location of alkylresorcinols (ARs) in cashew nut fruit and cereal caryopses. Cross sections (a) of a cashew fruit showing the nut shell where ARs are contained (adapted from De Lima *et al.*, 2008), and (b) of a cereal caryopse showing the layer of ARs (violet staining with Fast Blue B) on the surface of the outer cuticle of the testa/inner cuticle of the pericarp (according to findings of Landberg *et al.*, 2008). (c) Natural non-isoprenoid phenolic lipids found in cashew nut liquid extracted from *Anacardium occidentale*. (**1**) anacardic acid; (**2**) cardols; (**3**) cardanols; (**4**) 2-methyl cardols.

two types: solvent-extracted immature CNSL (iCSNL) and technical CNSL. An iCNSL contains anacardic acid (60–65%), cardol (15–20%), cardanol (10%) and traces of 2-methyl cardol. Depending on the conditions of the roasting process, the composition of the technical CNSL can have higher cardanol content (83–84%), less cardol (8–11%) and 2-methyl cardol content (2%) (Fig. 10.4c). Components of CNSL have important applications in industry. In the case of gramineae, 5-n-ARs are present in high amounts (~0.03–0.15% of dry weight) in kernels of wheat (*Triticum aestivum*), durum wheat (*Triticum durum*) and rye (*Secale cereale*). In maize (*Zea mays*), ARs have been reported in amounts less than 5 µg/g (Gembeh *et al.*, 2001; Ross *et al.*, 2004c). In our laboratory, however, the search for ARs in a maize population with a broad genetic base suggests that these compounds, at least as aglycones, are completely absent in maize kernels. High levels of ARs are present in kernels of triticale (x triticosecale)

and various *Triticum* species other than *T. aestivum*, whereas low levels (40–100 μg/g) are found in barley (*Hordeum vulgare*). In cereals, AR are located in the outer layers of the kernel (Tluscik, 1978) and more than 99% of the AR content is present in the outer cuticle of testa/inner cuticle of pericarp (Fig. 10.4b) (Landberg *et al.*, 2008). This situation makes it possible to use ARs as biomarkers of cereal intake. In the past decade 5-n-ARs have been found in seedlings and plants of rice, sorghum and rye, reinforcing the hypothetical phytoanticipin function of these molecules. Interestingly, ARs were not found in grains of rice and sorghum, and further research may provide more evidence of the presence of ARs in seedlings and later phenological stages of cereal plants.

Several 5-n-ARs have been identified in strains of soil bacteria from *Azotobacter* and *Pseudomonas* families as well as in *Streptomyces* (Tsuge *et al.*, 1992), *Arthrobacter* and *Micrococcus* genus. The ARs occur in both vegetative and cyst forms (Kozubek *et al.*, 1996). An interesting feature of microbial 5-n-ARs is that the alkyl side chains are always saturated. Moreover, resorcinolic lipids were also found in fungal species from *Basidiomycetes* (Gianetti *et al.*, 1978) and *Hyphomycetes* (Stodola *et al.*, 1973), as well as in *Fusarium culmorum* (Zarnowski *et al.*, 2000a).

10.4 Extraction of ARs

As previously mentioned, ARs are insoluble in water, but are soluble in more hydrophobic solvents such as methanol, ethanol, acetone, ethyl acetate, diethyl ether, chloroform, cyclohexane and *n*-hexane. ARs are often extracted from cereal grains. Extraction is performed at room temperature from 1 g of cereal grains with 40 ml of acetone or ethyl acetate for 16–24 h, or from 25 g of grains extracted three times with 25 ml of acetone for 24 h each, and then filtered to remove solid particles (Kozubek and Tyman, 1999). Soxleth extractions for 2 h with acetone or cyclohexane were indicated as efficient as the last one (Zarnowski and Suzuki, 2004). Extractions with solvents more polar than acetone, such as methanol or ethanol, should be avoided because they co-extract other compounds that may interfere with later colorimetric and chromatographic analysis. Selectivity is in part achieved when whole grains are extracted, because ARs are only accumulated in the outer layers of the kernels. In our laboratory, extractions with acetone followed by flash micro-filtration on silica gel using hexane:chloroform:ethyl acetate (2:1:1, v/v/v) as mobile phase allow us to eliminate higher polar co-extracted metabolites, increasing the selectivity of the extracting method.

Extracting methods developed for ARs in cereal grains often have a low performance when applied to food products, where ARs sometimes may form inclusion complexes with other components, such as the starch in the bread, as occurs with other polar lipids (Ross *et al.*, 2004a). In such a situation ARs require more drastic extracting conditions. For example, complete recovery of AR from bread only was achieved using hot propanol:water (3:1, v/v), a method previously used for total lipid extraction from starch (Ross *et al.*, 2003b). This extraction method, however, is time consuming. Sometimes, the main purpose of AR analysis is to know the homologue composition more than the total content. In such cases, extracts from food products (i.e. pasta samples) obtained after continous stirring in ethyl acetate had the same composition of ARs as those originated from hot propanol:water extraction (Knödler *et al.*, 2009).

10.5 Rapid and Easy Methods for Detection/Preliminary Analysis of ARs

Several reasons have been presented on the need for efficient, fast and sensitive methods to analyse total content of ARs in cereal grains, including the use of ARs as biomarkers of whole grain or measure of ARs in breeding programmes of cereal crops.

Most general methods used for the determination of total AR content are based on spectrophotometry. The results obtained from these methods are usually calculated from appropriate calibration curves prepared on the basis of weight concentrations of a standard AR analogue and are expressed in μg/g dry matter (DM). Tluscik *et al.* (1981) developed a colorimetric method based on the use of the diazonium salt Fast Blue B BF4. The method was highly specific for 5-n-alkyl derivatives of resorcinols with a sensitivity of between 1 and 10 μg of ARs. Maximum absorbance of the coloured AR–Fast Blue B products was obtained at 520 nm after 1 h incubation at room temperature. Later, Gajda *et al.* (2008) improved this method replacing the Fast Blue BF4 (currently not commercially available) by Fast Blue B $ZnCl_2$ salt. This change lengthened to 3 h the stability of the products of reaction between the ARs and the diazonium salt. Sensitivity was also increased to 0.1 μg of ARs. Nevertheless, readings at 520 nm were only possible after 1 h of incubation, and formation of coloured products was reduced when exposed to sunlight. Fast Blue RR ½$ZnCl_2$ salt is often used for the detection of esterase and alkaline phosphatase activities in histochemical and colorimetric analysis (Johnston and Ashford, 1980). In these reactions naphthyl derivatives are employed as substrates and the enzymatic release of naphthol is followed via a coupling reaction with a diazonium salt such as Fast Blue RR ½$ZnCl_2$. These reactions are usually performed in basic media and the formation of coloured derivatives takes only a few minutes. This situation led us to develop a colorimetric method for measuring ARs based on the use of Fast Blue RR ½$ZnCl_2$ as reagent (Sampietro *et al.*, 2009). The sensitivity of the new method is comparable to that obtained in the Fast Blue B based method, but incubation time needed to complete the reaction has been reduced to 20 min and the stability of the coloured products is as long as 3 h, allowing the fast processing of large sets of samples.

Thin-layer chromatography (TLC) allows rapid qualitative detection and isolation of AR. TLC is often developed on silica gel 60, using mobile phases such as methanol:water (90:10, v/v), chloroform:ethyl acetate (9:1, v/v), benzene:ethyl acetate (85:15, v/v) (Kozubek and Tyman, 1999), chloroform: methanol (85:15, v/v) (Reusch and Sadoff, 1979), chloroform:methanol (4:1, v/v) or hexane:ethyl acetate:chloroform (2:1:1, v/v/v) as in our laboratory. In these systems, however, AR homologues are not resolved to appear as a single spot. Impregnation of the silica TLC plate with 20% silver nitrate in 50% methanol, and developing with benzene:ethyl acetate (85:15, v/v), separates AR on the basis of unsaturation, whereas impregnation of the silica TLC plate with 5% paraffin oil in *n*-hexane and development with acetone:methanol:water (60:15:25, v/v/v) allows separation on the basis of chain length (Kaczmarek and Tluscick, 1984). Alkyl saturated AR homologues also can be separated on Silica Gel Si60 RP-18 plates developed with methanol:water (95:5, v/v) (Rejman and Kozubek, 2004).

Several spray reagents can be used to detect ARs on TLC plates. In our lab they turn a red colour with vanillin–HCl reagent, bright-red colour with p-anisaldehyde–sulphuric acid reagent and pink to deep crimson with Fast Blue B $ZnCl_2$ salt. This last reagent is the most preferred and was shown to be the most sensitive for the visualization of ARs (Sampietro *et al.*, 2009).

10.6 GC–MS Analysis of ARs

Gas chromatography (GC) is often used for the quantitative determination of ARs because it allows rapid analysis with good separation of AR homologues (Ross *et al.*, 2004a). These compounds can be analysed without derivatization. Although conversion of ARs to their trimethylsilyl (TMS) ethers allows reduction of operation temperatures and lower retention times in GC, derivatization sacrifices fragmentation and structural information provided by underivatized ARs in mass spectrometry (MS) (Seitz, 1992). The ARs are usually separated on non-polar stationary phases, e.g. 100% dimethyl-polysiloxane

or 5% phenyl-methylpolysiloxane (Suzuki *et al.*, 1997). Several GC conditions have been used with or without split injection. In our laboratory, good resolution of ARs without derivatization was obtained using a capillary column Perkin-Elmer Elite-5MS (5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 µm film thickness), under the following conditions: 1 µl of the sample disolved in ethyl acetate (50 µl/mg) was injected to the column and a temperature gradient from 170 to 320°C (30 min) and 320°C (10 min). Helium was used as carrier gas at a flow of 1.2 ml/min, and split flow was adjusted to 80:1. Injector temperature was 280°C (Fig. 10.5).

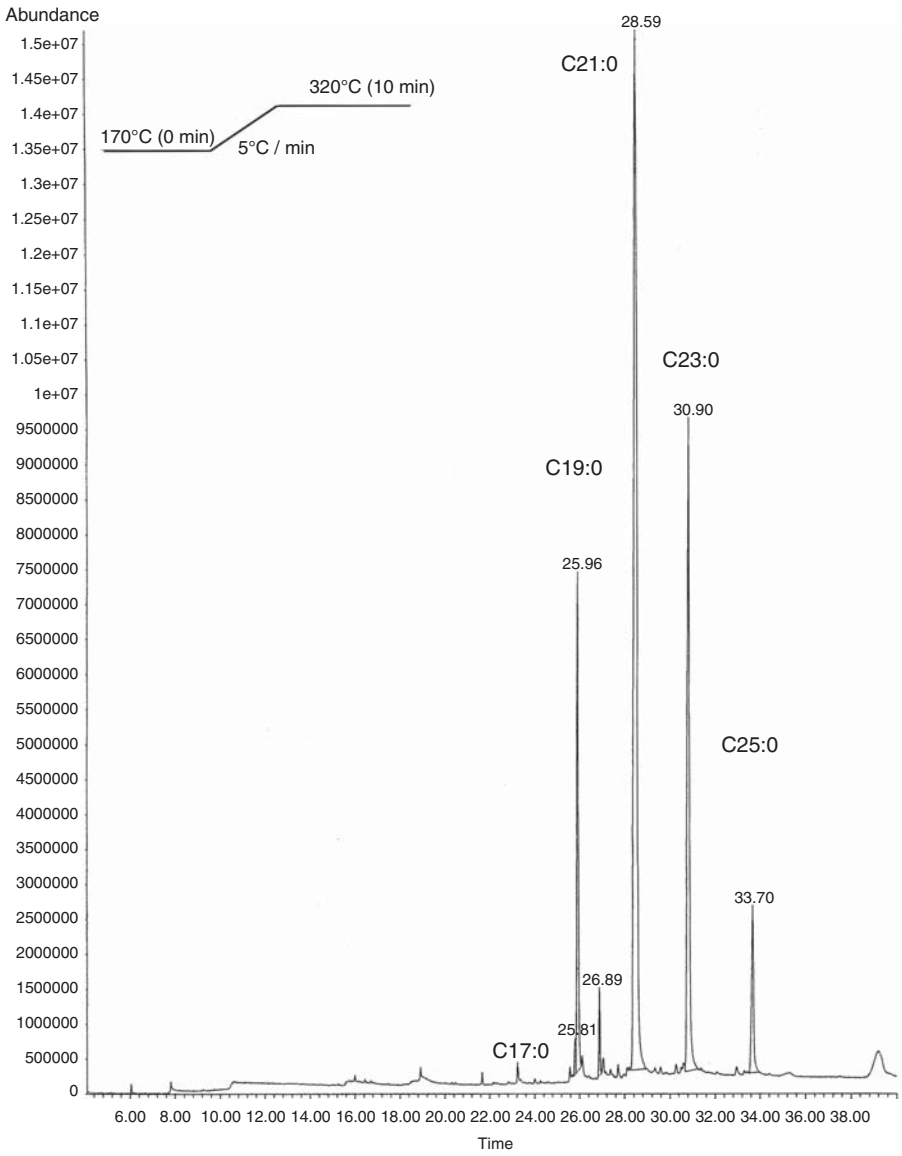


Fig. 10.5. GC–MS chromatogram of the alkylresorcinols (ARs) extracted from wholewheat grains. The ARs were injected without previous derivatization.

Total contents of ARs determined by gas chromatography and the colorimetric method based in Fast Blue B were highly correlated (Landberg *et al.*, 2009). In our laboratory we are currently comparing the accurancy of total levels of ARs determined by the new colorimetric method based on Fast Blue RR with those measured by GC–MS.

Electron impact mass spectra of ARs have a base fragment at *m*/*z* 124, due to McLafferty rearrangement of the phenolic ring, and other minor fragments at *m*/*z* 123 due to the dihydroxytropylium ion formed by direct β-cleavage, *m*/*z* 137 due to γ-cleavage and *m*/*z* 166 of unknown origin (Fig. 10.6). The *m*/*z* abundance ratio of 123/124 is about 1:5 in accordance with *meta*-dihydroxy substitution in the benzene ring.

High-performance liquid chromatography (HPLC) has also been used for the analysis of ARs. Prior to HPLC analysis, extracts may be filtered or purified by solid-phase extraction on silica, C8 or C18 and chromatography is achieved using reversed-phase columns. Detection coupled to HPLC has been performed with either UV or diode array detectors set at 275–280 nm. Absorption at these wavelengths was attributed to the resorcinolic ring (Ross *et al.*, 2004a). Nevertheless, current GC methods are superior to HPLC in their ability to separate different AR homologues. HPLC analysis offers possibilities for purification more than quantitative measurement and may be more appropriate for analysis of the more water-soluble AR metabolites in biological fluids.

10.7 Bioactivity in Organisms and Potential Roles for Resorcinolic Lipids in Plant Physiology

The amphiphilic character of 5-n-ARs has been repeatedly invoked as a key feature in the explanation of the biological activities of these lipid phenolics. Possible effects of ARs on living organisms are as follows.

10.7.1 Effects on nucleic acids

ARs can affect the structure and metabolism of nucleic acids. They are able to inhibit both DNA and RNA synthesis (Gianetti *et al.*, 1978), as well as to induce DNA strand scission (Scanell *et al.*, 1988). This process occurs in the presence of both copper and ARs (Singh *et al.,* 1995). A hydroxylation of the 5-AR substrate dependent on Cu^{2+} and O_2 would generate 6-alkyl-1,2,4-trihydroxybenzenes. The catechol moiety of these molecules would be oxidized by Cu^{2+}, generating a quinone derivative with subsequent formation of reactive oxygen species and alkylation of the DNA strand. It was shown that DNA binding is mediated by the alkyl substituent of ARs and becomes more efficient as the length of the chain increases. Formation of H_2O_2 and - OH seem to be needed for DNA scission and DNA cleavage is not sequence specific. Further reseach indicated that ARs can inhibit a DNA-repair enzyme, DNA polymerase β, at fairly low concentrarions (IC_{50} = 14 μM), and bis-ARs are more efficient in DNA cleavage and inhibition of the mentioned enzyme than 5-ARs (Starck *et al.*, 2000). DNA polymerase β is believed to repair the damage caused by agents employed as anti-tumour agents, such as bleomycin and cisplatin. Preventing DNA reparation, ARs and their derivatives offer the possibility of increasing the effectiveness of these anti-tumour agents. Other reports also showed ARs as being active competitive inhibitors of the reverse transcriptase (He, 1990).

ARs are also able to reduce the mutagenic activity of some indirect mutagens, whereas they are less active on direct mutagens. Compared with anthocyanins, ARs were potent inhibitors of the rate and frequency of induced mutations in cultured lymphocytes. Later, the anticancer effects of ARs were attributed to the capability of these compounds to increase apoptosis in genotoxically damaged cells (Gasiorowsky *et al.*, 2001). Although anti-mutagenic and anti-tumoural bioactivites of ARs seem promising, more research is needed to determine the real impact of these molecules *in vivo*.

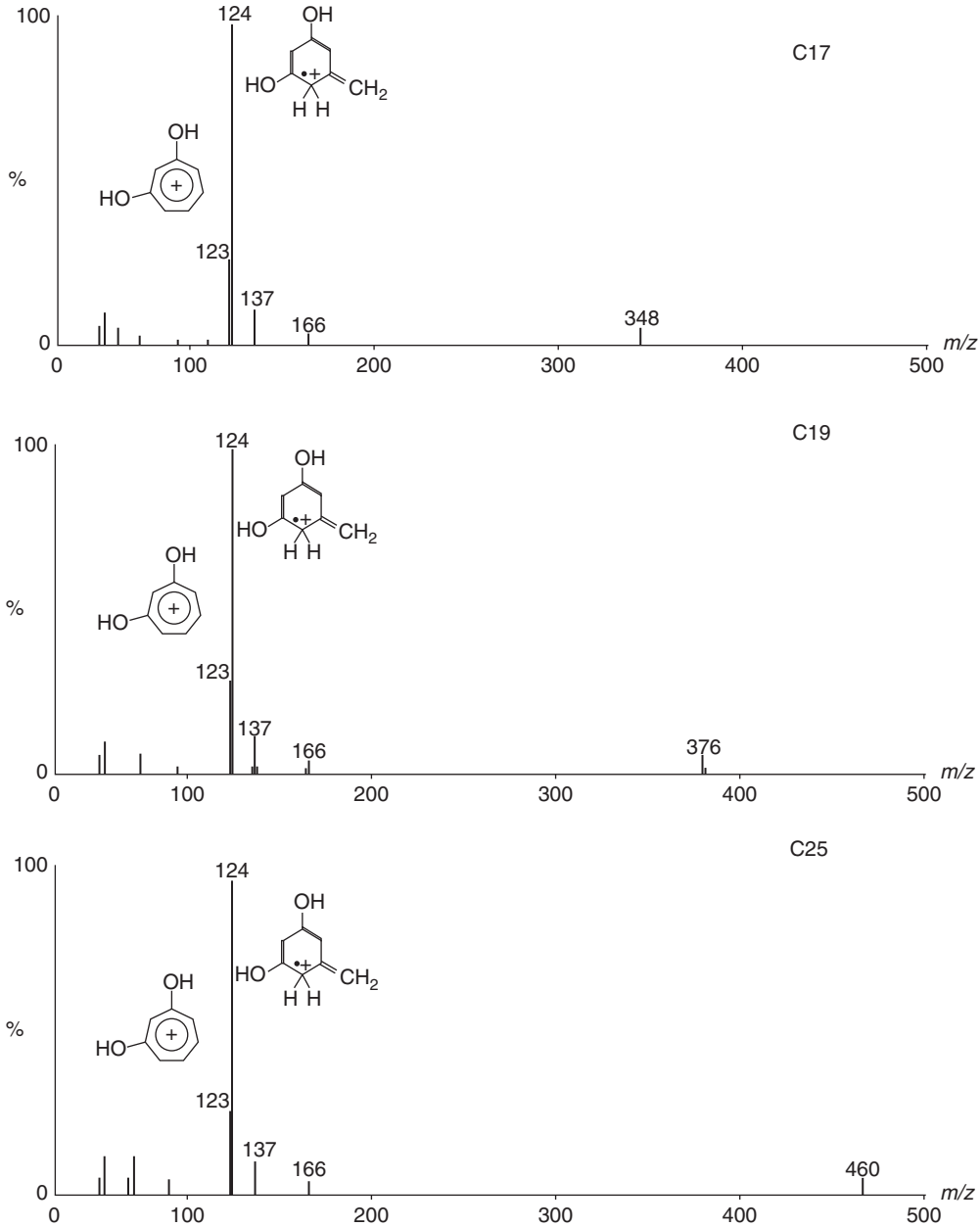


Fig. 10.6. Mass spectra of 5-n-alkylresorcinols homologues differing in the length of the alkyl chain. The typical fragmentation pattern is observed, including the base fragment at *m/z* 124 (McLafferty rearrangement of the phenolic ring) and fragments at *m/z* 123 (dihydroxytropylium ion).

10.7.2 Effects on proteins

ARs are able to inhibit the activity of several enzymes. This effect may be mediated by direct binding of these compounds to the hydrophobic regions of the proteins, particularly near tryptophan residues (Sikorski *et al.,* 1987). Another possibility could be the incorporation of ARs to a biological membrane, which can decrease the lipid

bilayer fluidity, as shown for phospholipase A (Gordeev *et al.*, 1991). The ARs also inhibited the activity of various dehydrogenase-type enzymes through interference with proton transport between co-enzyme and substrate/product, as occurs with NADH-dependent enzymes associated with respiration and photosynthesis (Nenashev *et al.*, 1989). In the latter case, the phenolic nature of resorcinolic lipids suggests that they may replace compounds such as ubiquinone or plastoquinone in mediating processes of electron transport (Rejman and Kozubek, 1997).

10.7.3 Effects on cell membranes

The ARs can easily be incorporated into the lipid bilayer of biological membranes where they induce a range of structural changes. In a model system ARs can affect phospholipid membranes differently depending on the way that they are added (Siwko *et al.*, 2009). The addition of ARs before the formation of phospholipid vesicles stabilizes phospholipid bilayer. In this situation the alkyl moiety would increase the order of lipid chains. Then, the membrane thickens, the interface dehydrates and the membrane becomes less permeable to water and solutes such as ions and glucose. In contrast, ARs disturb membranes, increasing the release of soluble markers when added to a suspension of already formed liposomes (Kozubek, 1987). In such a case ARs would generate transient water pores with an increase in leakiness. Longer alkyl chains and more unsaturated bonds enhance the dual effect of resorcinols on biological membranes.

10.7.4 Effects as chemical signals on plants and microorganisms

ARs may be chemical signals involved in plant–plant interactions. The relationship between maize, sorghum and the parasitic plant *Striga asiatica* (scrophulariaceae) is an example. The seeds of *S. asiatica* require a germination stimulus and, once germinated, *Striga* survives for less than 2 weeks in the absence of a host. A resorcinolic lipid derivative, xenognosin, isolated from roots of the host plant was able to promote *Striga* germination (Kato *et al.*, 1985). Further studies showed that its methylated form enhances the xenognosin-dependent germination (Orabi *et al.*, 1991). Another example is root exudates from rice. Allelopathic accessions produce higher levels of 5-n-AR glycosides than non-allelopathic accessions. Nevertheless, it is not clear if these compounds exert their action on other plants or regulate microbial populations in the rice rhizosphere. In this regard, the possible effect of ARs exuded from rice roots on soil bacteria was investigated using a set of biosensors that allows the monitoring of bacterial gene induction in real time (Miché *et al.*, 2003). This set consists of plasmids where the promoters of genes belonging to the predominant stress responsive networks are fused to the bioluminescence genes *luxCDABE* of the marine bacterium *Vibrio fischeri* (Meighen and Dunlap, 1993). Members of this set of plasmids have been described and proved to respond specifically to environmental-stress-inducing agents (Belkin *et al.*, 1997). It was shown that rice exudates actually exert a general stress on *Escherichia coli* sensor strains, inducing networks responding to protein damage and oxidative stresses but not to DNA damage. Moreover, the effects of alk(en)ylresorcinols extractable from the root surface of rice seedlings (Bouillant *et al.*, 1994) were comparable with whole exudates, suggesting that they may participate in the plant-induced selective pressure on microbial communities *in vivo*. It is important to note that the responses induced would be deleterious effects of ARs exerted on living membranes more than interactions of these molecules with specific membrane receptors. Other works also suggest that ARs are chemical signals in microbial interactions and the microbial producer would release ARs to the environment. In this case ARs would be autoinducers or autoregulators because the own microbial producer can respond to them. Accumulation of ARs up to a threshold concentration would reflect population density, which leads to a

concerted action of microbial cells (quorum sensing), as, for example, a general induction of anabiosis (Mulyukin *et al.*, 2001). Although atractive, the hypothesis of chemical signalling mediated by ARs needs further research, especially because soil abiotic factors can affect the integrity of AR molecules (e.g. by complexation or oxidation) before exerting their action on receptor organisms, avoiding the process of signal delivery (Kamnev *et al.*, 2009).

10.7.5 Phytoanticipin role

The fungicide, fungistatic and bacteriocide activities of ARs together with their constitutive expression in plants suggest a phytoanticipin function for these molecules (Kozubek and Thyman, 2005). The highest concentrations of ARs are in seedlings and young tissues but are also high during the entire plant growth. Glycosylated ARs may be produced in plant tissues to avoid autotoxicity and/or to facilitate transport into the plant or release to the environment. As observed for other phytoanticipins, the biosynthesis of ARs also can be increased by wounding, exposition to chemicals and biotic stimuli. Experiments *in vivo* on rye coleoptiles showed that the infection of the seedlings with *Fusarium culmorum* significantly increased the level of plant ARs (Kozubek *et al.*, 2001). Treatment of cereal grains with exogenous resorcinolic lipids results in increased plant resistance to various phytopathogenic fungi. These observations suggest that ARs participate in host–pathogen relationships.

10.8 ARs and their Derivatives Isolated from Medicinal Latin American Plants

With the exception of the cashew nut, reports on the isolation, identification and characterization of ARs from cultivated or medicinal Latin American plants are scarce and often involve AR derivatives. For example, *Lithraea molleoides* is a tree naturally growing in South America, commonly known as 'chichita' or 'molle Córdoba' in Argentina. Its leaves are used by rural people as an anti-arthritic, haemostatic, diuretic and tonic, and in the treatment of respiratory diseases. As occurs with other Anacardiaceae species, phenolic lipids in *L. molleoides*, as well as in the related species *Lithraea brasiliensis*, were responsible for allergic contact dermatitis caused by catechol-type compounds rather than ARs (López *et al.*, 2005). This plant is a source of 1,3-dihydroxy-5-(tridec-4′,7′-dienyl)benzene, an AR derivative that induces apoptosis in human tumoural cell lines (Barbini *et al.*, 2006). *Lithraea caustica*, commonly known as 'litre', is an endemic Chilean species of the same genus (Russo *et al.*, 2009). Its stem juice is used for cough treatment. Litreol, the active compound identified from their leaves, has been shown to be a 3-[pentadecyl-10-enyl- catechol] instead of an AR. This lipid phenolic inhibits cancer cell viability in a dose-dependent manner and induces apoptotic cell death at 0.59–1.18 μM. Another Anacardiaceae, the Mexican *Amphipterygium adstringens*, is traditionally used in the treatment of gastritis and ulcers (Castillo-Juarez *et al.*, 2007). It contains alkylphenolic acids (C15:0, C16:0, C17:0 and C19:0) in its bark. These compounds showed potent activity against *Helicobacter pylori*, the major etiological agent of chronic active gastritis and peptic ulcer disease, linked also to gastric carcinoma. The leaves of a Mexican Myrsinaceae, *Stylogyne turbacensis*, contain 5-[11′(*S*)-hydroxy-8′-heptadecenyl]resorcinol and 5-[12′(*S*)-hydroxy-8′,14′-heptadecadienyl]-resorcinol (Jimenez Romero *et al.*, 2007). These compounds showed a strong activity in the leishmania assay at 7 and 3 μM, respectively. The 5-[12′(*S*)-hydroxy-8′,14′-heptadecadienyl]-resorcinol showed moderate activity against a drug-resistant strain of *Trypanosoma cruzi* with an IC_{50} value of 22 μM. The cushion herb *Oxalis erythrorhiza* (Oxalidaceae) is known as 'boldo de la cordillera' in the San Juan province of Argentina. A decoction of the aerial parts is recommended for hepatic and heart ailments (Feresin *et al.*, 2003). The aerial parts contain 3-heptadecyl-5-methoxyphenol and the lipidic quinone embelin. The former compound was active against

Leishmania amazonensis and *Leishmania donovani* promastigotes with 100% lysis at 100 µg/ml. The cytotoxicities (IC_{50}) of embelin and the mentioned AR on human lung fibroblasts were 739 and 366 µM, respectively. Embelin was the main active constituent isolated. Because *O. erythrorhiza* is used to treat heart complaints, a symptomatology related to Chagas' disease, a possible link between the traditional use of the plant extract and the trypanocidal effect was suggested. Phenolic lipids associated with the antioxidant activity were also detected in *Chenopodium pallidicaule*, an Andean pseudocereal (Peñarrieta *et al.*, 2008).

10.9 Potential Uses of ARs in Agriculture

The accumulation of ARs in cereal plants may increase their resistance against noxious organisms. This should reduce the input of, and dependence on, synthetic agrochemicals (Gealy *et al.*, 2003). Several strategies were proposed to achieve a high content of ARs in cereal plants, as described below.

10.9.1 Breeding improvement

Although the determination of AR content was suggested as a valid indicator of disease resistance in cereal grains (García *et al.*, 1997), there is no breeding programme oriented to select crop varieties with enhanced AR accumulation. Most knowledge on AR content and composition is currently restricted to cereal kernels. AR composition is species dependent, whereas both environment and cultivars determine the total content (Ross *et al.*, 2003b, 2004a; Zarnowski and Suzuki, 2004). Root exudation of AR glycosides and derivatives (i.e. sorgoleone) was related to rice and sorghum allelopathy, respectively (Kong *et al.*, 2002; Dayan *et al.*, 2005). Identification and characterization of enzymes involved in the biosynthesis of these metabolites are in progress. Nevertheless, much remains to be done to fully understand not only the synthesis of ARs but also the factors regulating their production, which may allow the possibility of transgenic manipulation of the lipid resorcinol pathway (Dayan *et al.*, 2005).

10.9.2 Promotive biotic and abiotic factors

Biotic and abiotic factors can induce the synthesis of phenolic compounds, which is sometimes related to activation of responses in plant defence (Daniel *et al.*, 1999). This premise led to *in vitro* assays where the influence of physical and chemical factors was evaluated on the accumulation of ARs in rye and rice. Seedlings of these cereals grown under light accumulate less ARs than those kept in the dark (Suzuki *et al.*, 1996; Magnucka *et al.*, 2007a). As previously mentioned, the synthesis of ARs was related to mitochondrial and plastidial compartments, and higher contents in etiolated plants were attributed to a higher number of plastids in etiolated seedlings (Deszcz and Kozubek, 2000). The decrease in temperature stimulates the accumulation of ARs in rye seedlings, with a higher participation of unsaturated ARs. Seedlings grown in solutions containing fungicides (benomyl or Carbendazin) or herbicides (lenacil, chloridazon or norflurazon) provided at 10 ppm enhanced the levels of ARs, although it was not clear how these biocides exert their effect on AR metabolism (Magnucka *et al.*, 2001; Magnucka *et al.*, 2007a,b). These results are promising, but further research is needed in field conditions to know the real impact of these chemicals on AR contents in rye.

The synthesis of ARs in response to biotic factors was also investigated in barley. Barley grains were inoculated with *Pseudomonas fluorescens* PsR21 before sowing in field plots (Zarnowski *et al.*, 2000b). Inoculation significantly increased the crop yield at harvest, compared with control plants. Infections with phytopathogenic fungi were apparently decreased. Both control and inoculated plants contained comparable amounts of ARs. Plants treated with the bacterium as well as control plants biosynthezised the same homologues with

carbon side chains from C_{17} to C_{25}. However, the relative content of the short-chain alkylresorcinols (C_{17} and C_{19}) in inoculated plants was lower and that of the longest homologue (C_{25}) higher, suggesting that the inoculated plants may be more efficient against pathogen attack.

10.9.3 Improvement of biological control through liposomal technology

In the near future, liposome technology could be combined with biological control by using liposomal vehicles with built-in AR molecules (or AR derivatives) and/or bacterial cells entrapped inside (Kozubek *et al.*, 2000).

10.10 Quality of Cereal Products and AR Composition

As previously mentioned, ARs are abundant in the outer layers of rye, wheat and barley grains. Then, they are only present in foods prepared with whole grains or in the bran of these cereals. The ratio of the homologues C17:0 to C21:0 was shown to be up to 0.02 for the whole grain of durum wheat and associated products, whereas it was 0.1 and 0.9 for common wheat and rye, respectively (Landberg *et al.*, 2005). This ratio has been proposed to indicate the source of grains in foods. Such knowledge has allowed the development of methods for the detection and quantitation of the adulteration of whole-cereal flours and pastas (Knödler *et al.*, 2009).

10.11 Resorcinolic Lipids as Biomarkers of Whole-grain Intake

Epidemiological studies strongly suggest a link between the consumption of whole-grain cereals and decreased risk of heart disease, diabetes, obesity and certain cancers (Åman *et al.*, 2007). Elucidation of the relationships between whole-grain diets and health is incomplete because of the lack of specific biomarkers of whole-grain intake (Asp and Contor, 2003). Dietary biomarkers are compounds that can be measured in a biological sample (e.g. adipose tissue, plasma or urine) and can be non-subjectively related to the intake of a specific food/food group, which may be linked to a biological activity and/or decreased risk of disease. As previously mentioned, several investigations suggest that ARs are biomarkers for human whole-grain intake (Ross *et al.*, 2004c). ARs are absorbed from the small intestine via the lymphatic system and are incorporated into human erythrocyte membranes (Ross *et al.*, 2003a). Plasma ARs are transported in lipoproteins, with very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) being the main carriers. Then they can be determined quantitatively in the serum and also their metabolites in human urine (Linko-Parvinen *et al.*, 2007). They have also been detected in the adipose tissue of rats (Ross *et al.*, 2004b). Recent studies have shown that AR can inhibit LDL oxidation *in vitro* and might provide antioxidant protection when present in biological membranes (Kozubek and Nienartowich, 1995). Further data have confirmed that cereal-grain 5-alkylresorcinols modify animal lipid metabolism *in vivo* and *in vitro* (Kozubek and Tyman, 2005), which make them very interesting in relation to the risk of diabetes, obesity, heart disease and some cancers.

10.12 Industrial Uses of Lipid Phenolics

Chemical uses of phenolic lipids from CNSL have attracted interest mainly because of the development of methods for their separation and the potential use of cardanol as a replacement for alkylphenols of petrochemical origin (Tyman, 1979). The CNSL obtained from cashew nutshell can be employed as a phenolic source for formaldehyde polymerization and can be used in compounded forms as a friction dust for automobile brakes and clutches. The phenolic lipids from CNSL also can be used in the synthesis of aryl glycolipids that, upon self-assembly, generate an array of soft materials such as lipid nanotubes,

twisted/helical nanofibres, low-molecular-weight hydro/organogels and liquid crystals, with potential applications in biomaterials, templated synthesis and biosensors fields (John and Vemula, 2006). Cardols can be used as the starting molecule for the synthesis of bioactive compounds, such as lasiodiplodin, a compound with plant growth regulating and anti-leukaemic properties (Dos Santos and Magalhães, 1999). The ARs have potential applications in the pharmaceutical industry, in the development of commercial liposome-based drugs. Liposomal vesicles have been used as carriers of various bioactive molecules. They markedly change the pharmacokinetics of a drug and lower its systemic toxicity, also preventing early degradation and/or inactivation after introduction to the target organism (Allen, 1997). Investigations showed that the presence of ARs and some of their semi-synthetic derivatives in liposomes allows the enhancement of liposomal drug encapsulation, reduces the amount of the lipid carrier necessary for efficient entrapment of anthracycline drugs, stabilizes liposomal formulation of the drug (both in suspension and in a lyophilized powder), does not influence liposomal fate in the blood circulation system and provides benefits from other biological activities of their resorcinolic lipid modifiers (Kozubek *et al.*, 2000).

10.13 Conclusions

Resorcinolic lipids have been extensively studied, not only from the chemical but also from the biological point of view. Interesting applications of ARs have been proposed in nutrition, agriculture and industry, with important impacts on human health. The physiological functions of ARs in plants need further research. The ARs may not only be phytoanticipins but also may regulate fluidity of cell membranes from plant producers. Research oriented to establish stress-induced changes in both fatty acids from phospholipids and AR levels/composition may provide a clearer comprenhension of the roles of ARs in plant physiology. In the coming years there is expected to be increasing interest in the bioactivities of both synthetic and natural AR derivatives, including those from Latin American plants. Further interdisciplinary research is needed to create a full picture of ARs biosynthesis, physiological roles and potential practical applications.

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11 Phytoecdysteroids and Related Sterols Isolated from Mexican Cacti: their Potential Use as Natural Insecticides

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11.1 Introduction

The chemistry and structural diversity of sterols is very rich. Because sterols have been recognized in several biochemical reactions, research in the field is still growing. In recent years several novel structures and biological activities have been published, and new functions are now being recognized for those 'old molecules' sometimes even ignored in the past.

Sterols are present in fungi, plants and animals. Campesterol and sitosterol are the major end-products of the sterol pathway in higher plants (Schaller, 2003). Most insects require sterols in their diets; cholesterol is the major sterol found in insects. It serves as a structural component of cell membranes and as the precursor of the insect moulting hormones, ecdysteroids. Cholesterol has been shown to support normal development in most insects and it will satisfy the dietary need for sterol in most insects (Canavoso *et al*., 2001). Although most plant material contains little or no cholesterol, most phytophagous insects are capable of obtaining adequate cholesterol by converting C_{28} and C_{29} phytosterols to cholesterol via dealkylation of the C-24 alkyl group (Svodova, 1999).

The nature of the relationship between phytophagous insects and the plants they eat has been studied extensively but, even today, many questions still remain unanswered. Some points that remain are: (i) knowledge of the quantity and nature of the sterols present in plants; (ii) the exact mechanisms through which the insects use such mixtures of sterols in their diets; and (iii) the possible uses of sterols in the context of plant–insect relationships and as pharmaceuticals, and so on.

In this chapter we review some aspects of the chemistry of ecdysteroids, relating it to the chemistry of the sterols present in the family Cactaceae.

11.2 Ecdysteroids: a Family of Peculiar Sterols

Ecdysteroids are a group of polyhydroxylated steroids that act as hormones in all arthropods, studied primarily in insects. These steroids are present at all stages of insect development, regulating many biochemical and physiological processes such as embryonic and post-embryonic developments, moulting and metamorphosis, reproduction and diapause (Dinan, 2001).

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The first ecdysteroid isolated was ecdysone (25 mg) from pupae (500 kg) of the silk worm *Bombyx mori*, using the *Calliphora* bioassay to direct the isolation (Butenandt and Karlson, 1954). The poor yield, which represents a purification factor of about $1:10^7$ of the hormone, as well as the absence of modern physical techniques, delayed the structural elucidation of this compound (Karlson, 1996). The full structural elucidation of ecdysone (**1**) was achieved in 1965, by a combination of chemical experiments (Karlson *et al.*, 1965) and X-ray analysis of the crystals (Huber and Hoppe, 1965).

Since then many ecdysteroids have been found in arthropods, such as 20-hydroxyecdysone (**2**), recognized as the most important ecdysteroid in insects, as well as ponasterone A (**3**), 2-deoxyecdysone (**4**) and 25-deoxyecdysone (**5**), among others (Fig. 11.1). The principal structural feature of ecdysteroids, the 14α-hydroxy-7-en-6-one chromophore, is derived from cholesterol (**6**). For this reason, most of them have C_{27} cores.

There is, however, some diversity in the number of carbons of ecdysteroids depending on the alimentary habits of species and their metabolic pathways. The phytophagous insects include in their diets C_{28} and C_{29} sterols such as β–sitosterol (**7**), stigmasterol (**8**), campesterol (**9**) and fungal ergosterol (**10**). In some insects, those phytosterols can be metabolized to synthesize cholesterol and then be used as a hormone precursor to form C_{27} ecdysteroids (Fig. 11.2). They can dealkylate C_{28} or C_{29} phytosterols. On the other hand, if the insects are not

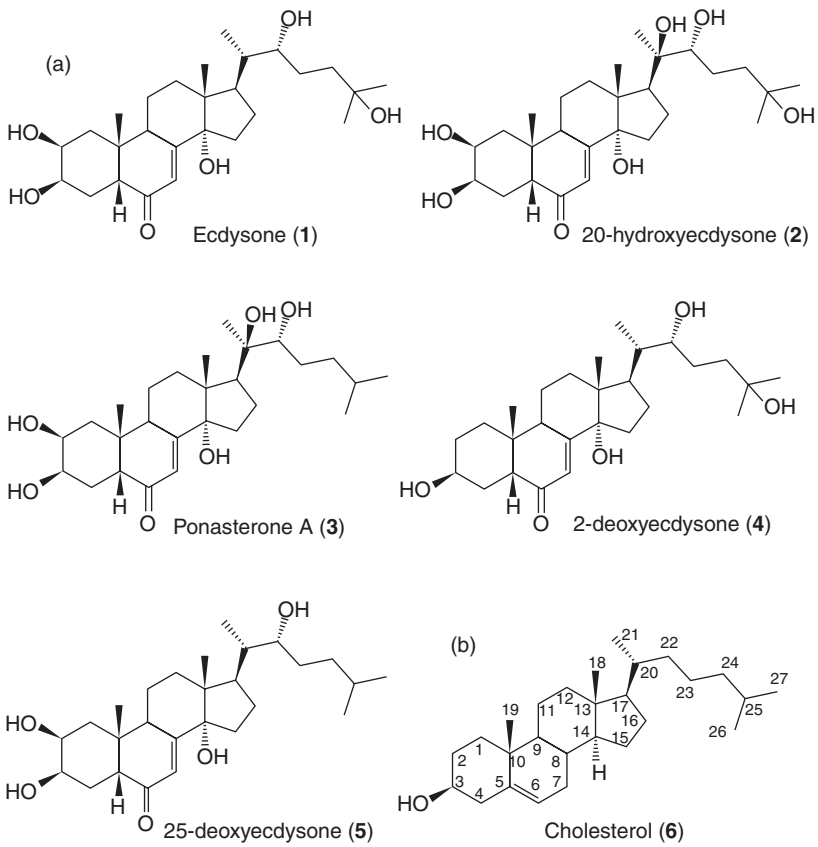


Fig. 11.1. (a) Chemical structures of some of the most commonly occurring ecdysteroids in insects. (b) Cholesterol, their chemical precursor.

capable of making the dealkylation, they can synthesize C_{28} or C_{29} ecdysteroids (Shaaya, 2008; Lafont & Koolman, 2009).

Although the ecdysteroids are structurally diverse, they are expected to have a set of minimum structural requirements to carry out their biological activity. The structural requirements for most biologically active ecdysteroids include: (i) a *cis* A/B ring junction (5β-H); (ii) a 7-en-6-one group; (iii) a complete sterol side chain with a 22R oxygen function; (iv) an oxygen function generally in the form of a 3β-OH group; and (v) additional OH groups at C-14α and C-2β, and, in many cases, also at C-20 and C-25 (Dinan, 2001). Despite these structural characteristics, there are several examples of biologically active substances but with some changes in the patterns of hydroxylation and configurations in ring junctions, including the A/B ring junction.

Interestingly, almost on par with the discovery of ecdysone from silk worms, similar molecules were isolated from plants. Some of the first ecdysone-like compounds isolated from plants (Fig. 11.3) reported in the literature were viperidinone (**11**), viperidone (**12**) and deoxyviperidone (**13**), all from *Wilcoxia viperina* (Djerassi *et al.*, 1964; Knight *et al.*, 1966), a plant from Mexico belonging to Cactaceae, now named *Peniocereus viperinus* (Arias *et al.*, 2005).

Moreover, the first truly ecdysteroid molecules were isolated from two species of *Podocarpus*: 20-hydroxyecdysone (**2**) from the Australian brown pine, *Podocarpus elatus*

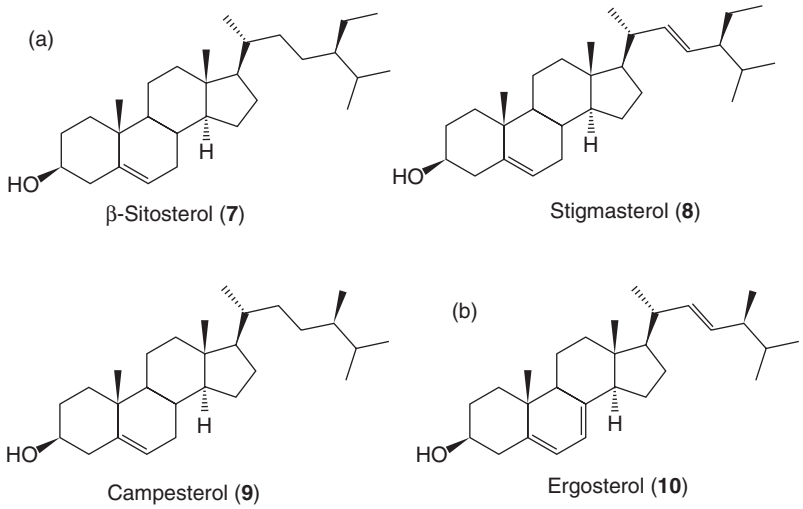


Fig. 11.2. Most common (a) plant and (b) fungal sterols. Some phytophagous arthropods and non-arthropod vertebrates have to consume these sterols in their diets, being able to synthesize ecdysteroids from this source.

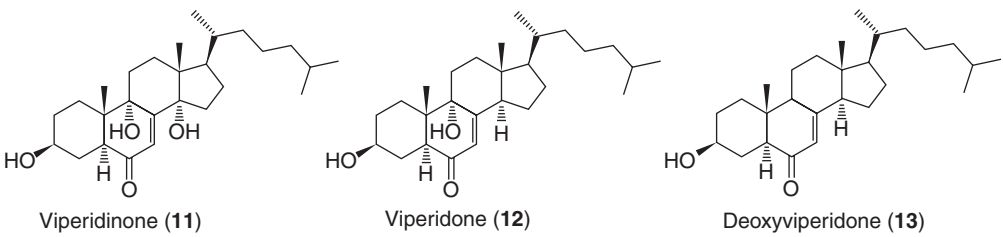


Fig. 11.3. Structures of ecdysteroid-like compounds isolated from Cactaceae.

(Galbraith and Horn, 1966), and ponasterone A (**3**) from *P. nakaii* (Nakanishi *et al.*, 1966), among others.

Since then more than 300 related compounds have been isolated, from species belonging to both divisions of terrestrial plants: Pteridophyta and Espermatophyta. Among the reduced number of plants that have been screened for ecdysteroids, the most common phytoecdysteroids found until now are 20-hydroxyecdysone (**2**), polypodine B (**14**), ponasterone A (**3**) and makisterone A (**15**) (Rharrabe *et al.*, 2010). It should be emphasized that compounds **2** and **3** are present in both groups of organisms, animals and plants (Fig. 11.4).

Unlike the structural diversity of ecdysteroids of animal origin, plants also have, in addition to the C_{27}, C_{28} and C_{29} ecdysteroids (Fig. 11.5), some C_{19} (Rubrosterone, **16**; dihydrorubrosterone, **17**), C_{21} (dihydropoststerone, **18**) and C_{24} (sidisterone, **19**) ecdysteroids (Báthori and Pongrácz, 2005). For full details of ecdysteroids in plants see Ecdybase (Lafont *et al.*, 2011).

The presence of ecdysteroids and similar compounds have been recorded in other organisms such as algae (Lafont *et al.*, 2010), fungi (Kovganko, 1999), non-insect arthropods and other non-arthropod invertebrates such as platyhelminths, nematodes, annelids, molluscs and tunicates, among other animals (Dinan and Lafont, 2007; Lafont and Koolman, 2009).

11.3 Phytoecdysteroids and Related Sterols from Cactaceae

The Cactaceae is a family of plants well adapted to arid environments, native to the Americas. In Mexico it represents the fifth

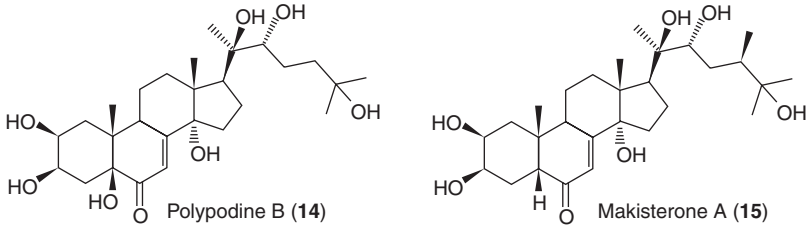


Fig. 11.4. Examples of ecdysteroids present in plants.

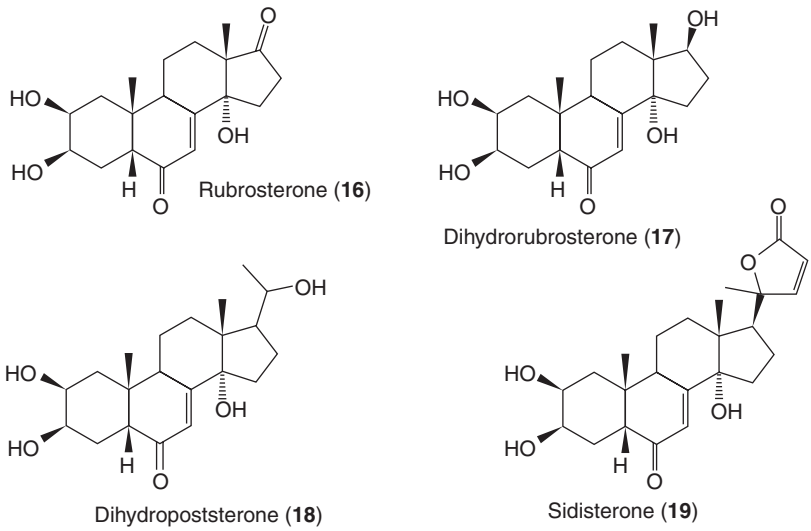


Fig. 11.5. Diversity of carbon number in phytoecdysteroids.

most diverse family and nearly 80% are endemic, distributed in almost all ecosystems, with more abundance in arid and semi-arid regions of central and northern states. Of the nearly 1500 species grouped in the family, very few have been studied on a chemical level. According to the data accumulated in the literature, the family is rich in triterpenes, alkaloids, phenolic compounds, betalains and sterols (Bravo-Hollis, 1978; Anderson, 2001).

As we noted, the history of sterol chemistry from Cactaceae has been linked closely with ecdysteroids and some related compounds were isolated early. Knight *et al.* (1966) mentioned that they had found viperidinone (**11**), the first molecule of vegetal origin with a structure similar to that of ecdysone, in the cactus *Peniocereus viperinus* (before *Wilcoxia viperina*). Viperidinone is 5α-cholest-7-en-6-one- 3β, α9, 14α-triol, with a *cis* A/B ring junction and without the characteristic hydroxylation pattern of ecdysone. The other compounds that were isolated, viperidone (**12**) and deoxyviperidone (**13**), both analogues of viperidinone, have one and two hydroxyl groups fewer, respectively.

Moreover, some sterols related to the structure of ecdysteroids have been isolated from Cactaceae (Figs 11.6 and 11.7). The sterols lophenol (**20**), schottenol (**21**), 24-methylenelophenol (**22**), lathosterol (**23**), 5α-campest-7-en-3β-ol (**24**), spinasterol (**25**), all of them with a double bond at C-7, together with locereol (**26**) and 5α-cholesta-8,14-dien-3β-ol (**27**), were isolated from *Lophocereus schotii* (Djerassi *et al.*, 1958; Campbell and Kircher, 1980). The 3β, 6α-dihydroxysterols peniocerol (**28**) and macdougallin (**29**), together with small amounts of lophenol, campesterol, β-sitosterol and cholesterol, were obtained from both *Peniocereus fosterianus* and *Peniocereus macdougallii* (Djerassi *et al.*, 1961, 1963, 1965). Lophenol and macdougallin are very peculiar because they have an extra α-methyl group on the cholesterol core, the former at C-4 and the latter at C-14.

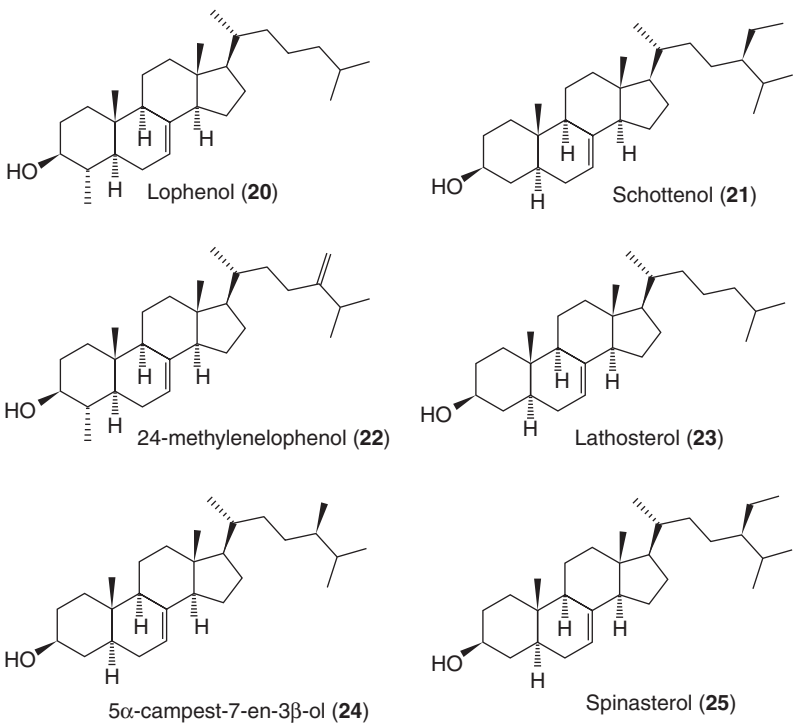


Fig. 11.6. Chemical structures of delta-7 sterols isolated from Cactaceae.

Furthermore, Knight and Pettit (1969) investigated *Peniocereus greggii* to find other 14α-methyl sterols, but failed. They did, however, isolate peniocerol, desoxyviperidone, viperidone, viperidinone, peniocerol and β-sitosterol, together with an unusual new 5β-desoxyviperidone (**30**) as the acetate derivative. Because they argued that the novel compound was possibly an artefact obtained during the isolation, they did not characterize it further.

Although this conclusion cannot be modified, it is notable that Jiang *et al*. (2006) have recently isolated two sterols, opuntisterol (**31**) and opuntisteroside (**32**), both with a 5β configuration, together with the known sterols β-sitosterol, 7-oxositosterol, 6β-hydroxystigmast-4-en-3one and daucosterol, from the aerial parts of the cactus *Opuntia dillenii*. Even though opuntisterol and opuntisteroside do not have a double bond at C-7, their discovery presents the possibility of finding molecules with the same 5β configuration, similar to ecdysteroids, in other plants of Cactaceae (Fig. 11.8).

Studies on the organ pipe cactus, *Stenocereus thurberi*, yielded a small amount of cholesterol, campesterol and sitosterol (Kircher, 1980), together with large amounts of peniocerol, macdougallin and three new 3β, 6α-dihydroxysterols, cyclostenol (**33**), stenocereol (**34**) and thurberol (**35**), the last two with a double bond at C-8 (Kircher and Bird, 1982) (Fig. 11.9).

Some sterols have been isolated from pollen from several species of Cactaceae (Fig. 11.10). In the pollen of *Carnegiea gigantea*, 24-methylene-cholesterol (**36**) was found to be the principal pollen sterol constituent, whereas some other minor sterols such as 24-dehydropollinastanol (**37**), β-sitosterol, cycloartenol (**38**), fucosterol (**39**) and 31-norcycloartenol (**40**) were

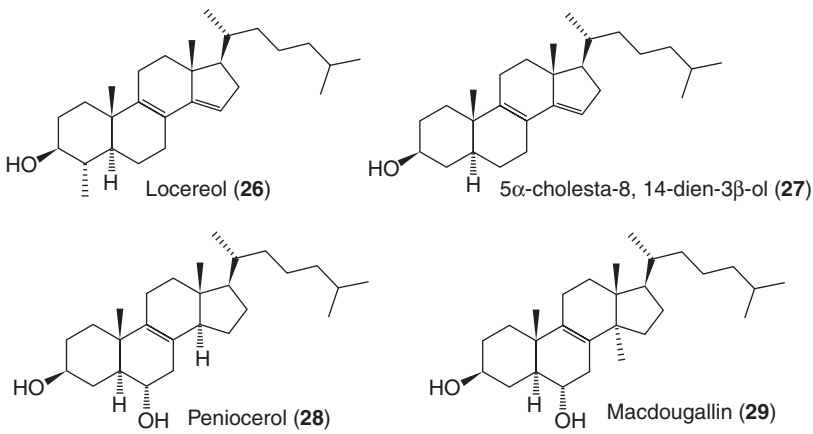


Fig. 11.7. Delta-8 sterols from Cactaceae.

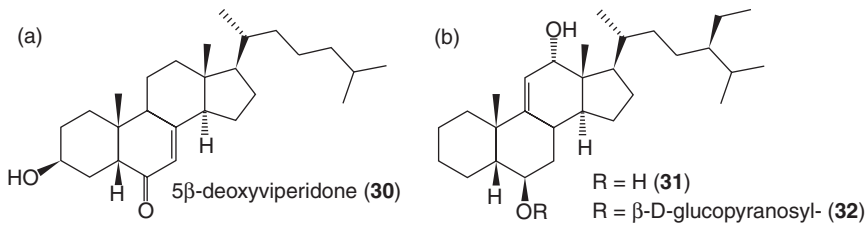
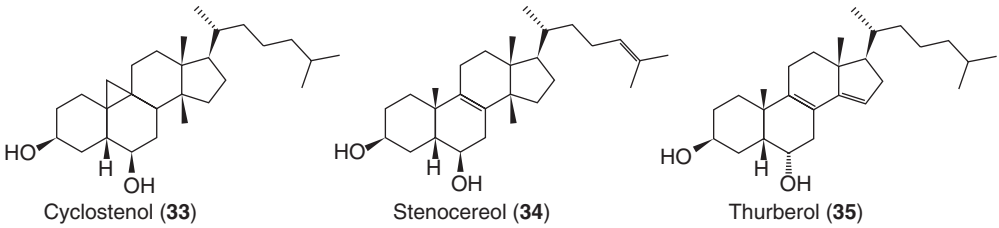
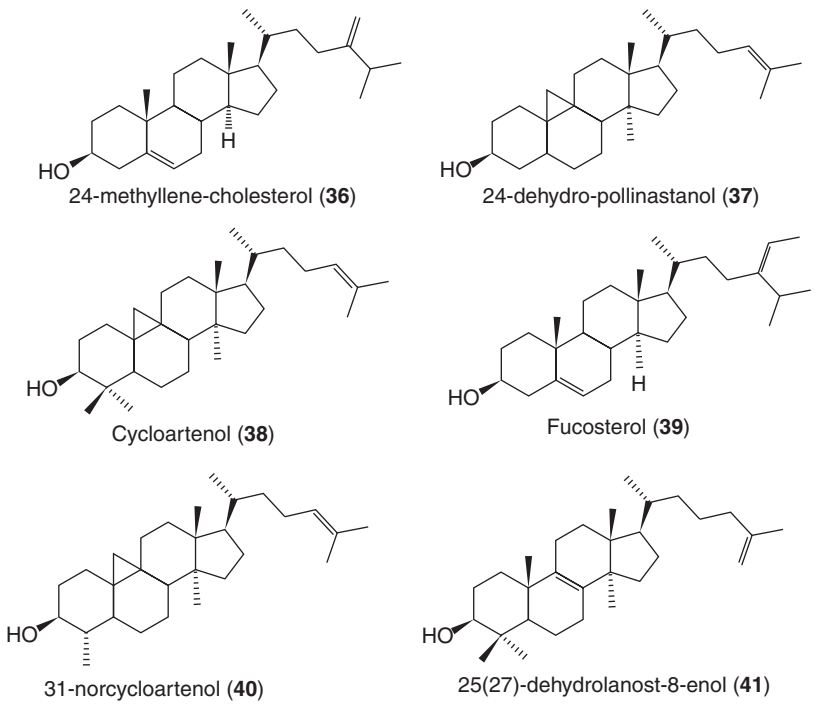


Fig. 11.8. 5β-Derivatives present in Cactaceae. (a) From *Peniocereus greggii*, the first 5β-cholesterol. (b) Opuntisterol (**31**) and opuntisteroside (**32**) from *Opuntia dillenii.*



Cyclostenol (**33**) Stenocereol (**34**) Thurberol (**35**)

Fig. 11.9. Sterols from the organ pipe cactus. The cholest-8-en-3,6-diol system is a special feature in Cactaceae.



24-methyllene-cholesterol (**36**) 24-dehydro-pollinastanol (**37**)

Cycloartenol (**38**) Fucosterol (**39**)

31-norcycloartenol (**40**) 25(27)-dehydrolanost-8-enol (**41**)

Fig. 11.10. Sterols from the pollen of Cactaceae.

found in *Opuntia phaeacantha* and *Opuntia versicolor*, and 25(27)-dehydrolanost-8-enol (**41**) was found in pollen of *Pachycereus pringlei* (Standifer and Barbier, 1968; Nes and Schmidt, 1988; Lusby *et al.*, 1993).

Finally, continuing with the survey of the chemistry of columnar cacti from Mexico, we have recently isolated peniocerol and macdougallin from roots of *Myrtillocactus geometrizans* (Céspedes *et al.*, 2005) and have evidence of the presence of other polyhydroxylated sterols from roots and aerial parts of this species (data not published).

The examples mentioned above only show some aspects of the chemistry of sterols presents in Cactaceae but were obtained in a very limited sample of species and tissues, not being representative of a particular clade nor for the whole family. With information available from samples of eight species of cacti (Salt *et al.*, 1987), plus those reviewed above, apparently the delta-5 sterols are the most representative of the family. There is, however, also a high prevalence of delta-7 and delta-8 sterols.

11.4 Potential Uses of Ecdysteroid-like and Related Sterols from Cactaceae

As far as we know, the biological activity of phytoecdysteroids from Cactaceae has not been investigated, even their possible agonist or antagonist ecdysteroid effects.

The chemistry of the triterpenes, alkaloids and sterols of some species of columnar cacti has been well documented (Kircher, 1969, 1980). The cactophilic fruit flies *Drosophila pachea, Drosophila nigrospiracula* and *Drosophila mojavensis* develop selectively in decaying tissues of *Lophocereus schottii* (common name senita), *Pachycereus pringlei* (cardón), *Stenocereus gummosus* (agria) and *S. thurberi* (organ pipe). It is known that sterols play a role in substrate selection and nutrition of the flies (Kircher *et al.*, 1984). *D. pachea* cannot develop unless it has an intake of delta-7 sterols in the diet, found only in *L. schottii*, its unique host. On the other hand, *D. nigrospiracula* larval success rate decreases when feeding on *S. thurberi* (organ pipe), which contains 3β,6α-diols such as peniocerol, whereas *D. mojavensis* can succeed in the organ pipe, its natural host (Fogleman *et al.*, 1986). It is therefore assumed that 3β,6α-diol sterols exert a defensive role in plants (Fogleman and Danielson, 2001). Recently we described the development of *Moneilema variolare* larvae, another cactophilic insect, in the roots of *Myrtillocactus geometrizans*, which is a new host to be reported for the insect (Salazar *et al.*, 2004). A chemical study was performed on the roots of *M. geometrizans*, resulting in the discovery of large quantities of mixtures of 3β,6α-diols. Peniocerol and macdougallin were the most abundant. In order to investigate the effects of both sterols against insects, *Spodoptera frugiperda* and *Tenebrio molitor* were selected as model systems. The results showed that peniocerol and macdougallin at low doses (5–50 ppm) inhibit insect moulting in *S. frugiperda* when incorporated into the artificial diet. When the larvae reached pupation, deformities were observed in the morphology of the pupae (Fig. 11.11). By contrast, when solutions of both the sterols were applied topically to *T. molitor* larvae, pupation was anticipated with respect to the solvent control. Both results indicate an effect on pupation, which is mediated mainly by 20-hydroxyecdysone. Thus, interference of the tested substances with ecdysone metabolism is strongly suggested (Céspedes *et al.*, 2005).

Fig. 11.11. Effects of peniocerol on *Spodoptera frugiperda*: deformation of pupae.

The above could be supported by the fact that it has long been known that derivatives of cholesterol with a double bond between C-8 and C-9 can be metabolized by rat liver homogenates to delta-7 cholesterol (Gaylor *et al.*, 1966). Moreover, peniocerol was converted by rat liver homogenates to cholest-7-ene-3β,6α-diol, presumably by way of 3β-hydroxy-cholest-7-en-6-one (Slaytor and Bloch, 1965). Although the above results involve mammal metabolism, no evidence was found in insect metabolism, so the proposal that peniocerol could be converted to the 7-en-6-one chomophore in insects has to be researched to try to explain the interference of moulting activities. These studies allow the proposal of ecdysteroids and related sterols found in Cactaceae as potential candidates for developing natural insecticidal agents.

Finally, another possible application of these compounds is due to their pharmacological activity. In a previous study, lophenol showed anti-tumour activity, and some derivatives were synthesized in order to obtain more bioactive compounds (He *et al.*, 2006). Furthermore, we evaluated the anti-tumour activity against some human cancer cell lines and anti-inflammatory activities of peniocerol and macdougallin. Both compounds showed moderate cytotoxicity against central nervous system carcinoma (U-251), prostate

carcinoma (PC-3), leukaemia (K-562), colon carcinoma (HCT-15) and breast cancer (MCF-7) human cell lines. Other experiments showed potent anti-inflammatory activity of peniocerol and macdougallin in induced-inflammation models (Salazar *et al.*, 2012). Taking into account the above, investigations on the pharmacological properties of these compounds should be continued to obtain more potent bioactive molecules from plants.

11.5 Conclusion

Sterols are an important group in the chemistry of most organisms. Plants synthesize different types of sterols, and only now do we begin to understand many of their biological functions. For phytophagous insects these substances play a key role because they represent the only source of obtaining hormone precursors such as cholesterol, which is converted into ecdysteroids. Because many plants contain substances similar to ecdysteroids, the chemical relationship between insects and plants becomes very complex. Several phytoecdysteroids and some related sterols isolated from plants could have a potential use as natural insecticides. We therefore need more studies in Cactaceae, which on the one hand will allow us to know whether other species of this family contain ecdysteroids that can be isolated and identified, and on the other hand will allow us to understand their possible uses as natural insecticides and as pharmaceuticals.

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12 *Zuccagnia punctata* Cav.: Phytochemistry, Traditional Uses and Pharmacology

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12.1 Introduction

Zuccagnia punctata Cav. (Fabaceae, Caesalpinioideae) is a monotypic endemic species that spontaneously grows in the arid and semi-arid regions of central and north-western Argentina, from the Mendoza to Jujuy Provinces. The vernacular names of this shrub are 'Jarilla macho', 'Puspus', 'Laca', 'Jarilla pispito' and 'Jarilla de la Puna'. This species together with other Leguminosae, Zygophyllaceae and Bromeliaceae are present in the phytogeographic province of 'Monte' in Argentina (Del Vitto *et al.*, 1997; Ulibarri, 1999, 2005). *Z. punctata* is a shrub of 1–5 m in height, is very aromatic because of a high content of resins in the foliage and grows in rocky and/or rubbly soils (Burkart, 1952).

12.2 Use in Traditional Medicine

Popular medicinal usage of *Z. punctata* is part of the indigenous traditional knowledge from north-western Argentina. This plant is used as a rubefacient, a foot antiseptic, against bacterial and fungal infections, against asthma, and as an anti-inflammatory and anti-tumoural agent, among others (Ratera and Ratera, 1980; Toursarkissian, 1980). Besides the traditional medicinal uses of *Z. punctata*, the plant is also employed as a fuel, in carpentry, in the construction of houses and in the manufacture of toys.

12.3 Phytochemistry

The first phytochemical studies on the composition of methanol extracts of aerial parts (leaves and twigs) of *Z. punctata,* erroneously referred to as *Larrea nitida*, resulted in the isolation of two chalcones, 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxy-chalcone (Fig. 12.1), and two flavanones, 7-hydroxyflavanone and 7-hydroxy-8-methoxyflavanone (Fig. 12.2) (Pederiva *et al.*, 1975). Recently, a new chalcone, 2′,6′-dihydroxy-4′-methoxychalcone, was isolated from aerial parts of the plant (Jimenez *et al.*, 2011) (Fig. 12.1).

3,7-Dihydroxyflavone and 3,7-dihydroxy-8-methoxyflavone were reported from leaf resin (Pederiva and Giordano, 1984). Of these, 3,7-dihydroxy-8-methoxyflavone was

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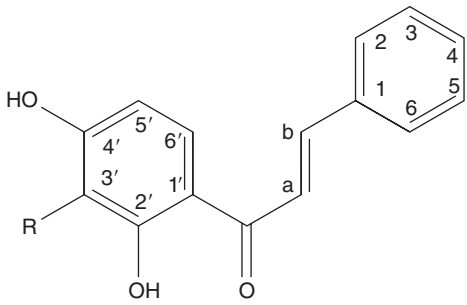

Fig. 12.1. Chemical structure of 2′,4′-dihydroxychalcone (R = H) and 2′,4′-dihydroxy-3′-methoxy-chalcone (R = OCH_3).

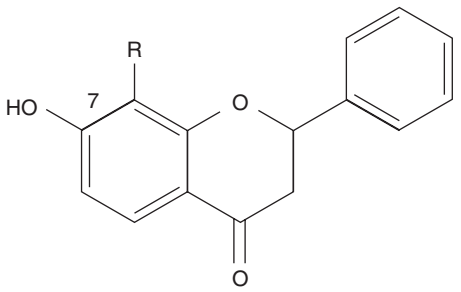

Fig. 12.2. Chemical structure of 7-hydroxyflavanone (R = H) and 7-hydroxy-8-methoxyflavanone (R = OCH_3).

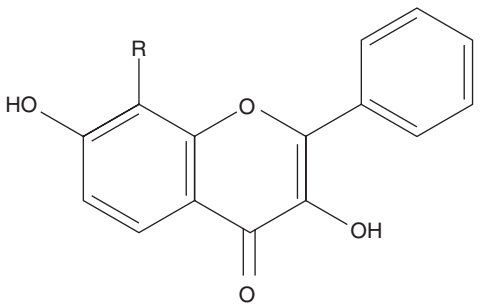

Fig. 12.3. Chemical structure of 3,7-dihydroxyflavone (R = H) and 3,7-dihydroxy-8-methoxyflavone (R = OCH_3).

a new natural product, whereas 3,7-dihydroxyflavone (Fig. 12.3) was a known compound reported earlier from *Platymiscium praecox* (Braga de Oliveira *et al.*, 1972).

The isolation of 3,7-dihydroxyflavone and 3,7-dihydroxy-8-methoxyflavone is of biogenic interest (Hahlbrock and Grisebach, 1975) because the biochemical precursor chalcones and flavanones, 2′,4′-dihydroxychalcone, 2′,4′-dihydroxy-3′-methoxy-chalcone, 7-hydroxyflavanone and 7-hydroxy-8-methoxyflavanone, were isolated from the same source (Pederiva *et al.*, 1975). The key steps in the formation of flavonoids is the condensation, catalysed by chalcone synthase, of three molecules of malonyl-CoA with an ester of coenzyme A and of a hydroxycinnamic acid, as a general rule *p*-coumaryl-CoA (the incorporation of caffeoyl-CoA seems quite exceptional, as the extra hydroxylation of the B ring occurs late in the process). In normal physiological conditions, chalcone tends to its cyclization in a reaction catalysed by chalcone isomerase, which induces a stereospecific closure of the cycle with formation of the basic structure of flavanones. To date, the mechanism of transformation of flavanones into flavones has not been elucidated. Moreover, the presence of 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone were also reported to occur together in the leaf resin of *Acacia neovernicosa* (Wollenweber and Siegler, 1982).

Two caffeic acid esters (Fig. 12.4; Fig. 12.5), 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate (**1**) and 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeate (**2**) (Svetaz *et al.*, 2004), together with 2′,4′-dihydroxychalcone, 2′,4′-dihydroxy-3′-methoxy-chalcone and 7-hydroxyflavanone were isolated from ethanol extracts of the aerial parts (leaves and twigs). The chemical synthesis of both caffeic acid esters was performed (Ramachandra and Subbaraju, 2006) starting from appropriate substituted benzaldehydes (Kavitha *et al.*, 1999; Venkateswarlu *et al.*, 2006). Hydroxy-substituted cinnamic acid esters are widely distributed in the plant kingdom and usually exist as esters of organic acids or sugars, or are bound to a protein and other cell wall polymers. Spectroscopy data of the synthetic propyl caffeates are in good agreement with those reported for the natural products (Svetaz *et al.*, 2004). Synthetic products were, however, obtained as optically inactive D/L-isomeric mixtures, whereas the naturally occurring compounds are L-isomers (**1**, $[\alpha]_D$: –27.0° (*c* 0.39, MeOH) and **2**, $[\alpha]_D$: –3.65° (*c* 0.25, MeOH)).

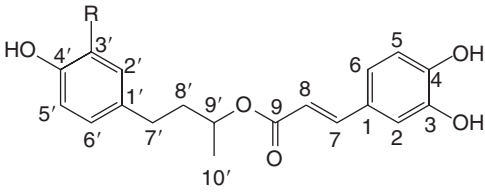

Fig. 12.4. Chemical structure of 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate (R = H) and 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeate (R = OH), according to Svetaz *et al.* (2004).

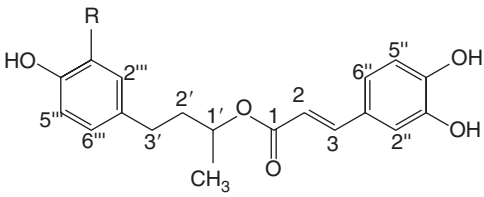

Fig. 12.5. Chemical structure of the synthetic propyl caffeates: 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate (R = H) and 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeate (R = OH), according to Ramachandra and Subbaraju (2006).

12.4 Bioactivities and Pharmacological Applications

12.4.1 Antibacterial activity

For thousands of years mankind has learnt about the benefits of plant use to alleviate or cure illnesses. The development of drug resistance and the appearance of undesirable side effects of certain antibiotics (Davies, 1994; Poole, 2001; WHO publication, 2001) have led to the search for new antimicrobial agents, mainly among plant extracts, in order to find new chemical structures to overcome the aforementioned disadvantages. Components of plants with antibacterial properties in general attack Gram-positive bacteria, whereas only some of them are active on Gram negative strains (Herrera *et al.*, 1996; Meng *et al.*, 2000; Scrinivasan *et al.*, 2001).

The antibacterial effect of the ethanol extract of *Z. punctata* aerial parts and of 2′,4′-dihydroxychalcone isolated from the same source on the growth of antibiotic-resistant Gram-negative bacteria was examined. Bacteria were clinical isolates from skin, blood and/or respiratory tract infections (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens, Morganella morganii, Acinetobacter baumannii, Pseudomonas aeruginosa and Stenotrophomonas maltophilia*) and ATCC reference strains (*Escherichia coli* ATCC 35218 and *Escherichia coli* ATCC 25922) (Zampini *et al.*, 2005). The selected bacteria are causal agents of infections in humans. The antibacterial activity was determined using the agar disc diffusion method (12.5–400 μg/disc); the minimal inhibitory concentration (MIC) was determined by serial agar macrodilution and broth microdilution assays (6.25–400 μg/ml) (NCCLS, 2002). MIC was defined as the lowest concentration of soluble principles or 2′,4′-dihydroxychalcone at which no colony was observed after incubation. The minimal bactericidal concentration was determined for concentrations of the extract or the purified substance at which 99.9 % of bacteria were killed.

Components of the ethanol extract were further separated by solvent extraction (ethyl ether) and column chromatography (Pederiva *et al.*, 1975; Zampini *et al.*, 2005), yielding, among others, a known compound (2′,4′-dihydroxychalcone) with a strong antibacterial activity (0.10–1.00 μg/ml) against *P. mirabilis, E. cloacae, S. marcescens, M. morganii, A. baumannii, P. aeruginosa* and *S. maltophilia*. These values are lower than that obtained for imipenem (0.25–16 g/ml), one of the most effective antibiotics against Gram-negative bacteria. Consequently, *Z. punctata* constitutes a good source of antibacterial compounds against Gram-negative resistant and multiresistant species. Many pharmacological properties attributed to chalcones would be ascribed to the ketone function of the three-carbon chain with an α,β-unsaturation. Substitutions on the A ring are most often identical to those of other flavonoids (2′, 3′, 6′). (This is equivalent to the 5- and 7-positions of the oxygen of the pyran ring. The numbering is reversed so that the benzophenone carbons are identified by

digits followed by the prime sign (′).) The B ring, however, is fairly unsubstituted. These types of compound are especially found in Fabaceae.

12.4.2 Antifungal activity

Fungi occur ubiquitously and are well adapted to use a wide range of substrates as their carbon, nitrogen and energy sources. These organisms can cause serious diseases in plants, animals and humans. Ethanolic extracts of leaves and twigs of *Z. punctata* showed fungicidal action against yeasts (*Saccharomyces carlsbergensis* and *Rhodotorula* spp.), wood-rot causing Basidiomycetes (*Rhodotorula* spp., *Lenzites elegans, Pycnoporus sanguineus* and *Schizophyllum commune*) and some phytopathogenic fungi (*Fusarium oxysporum, Penicillium notatum, Trichoderma* spp. and *Aspergillus niger*) (Quiroga *et al.*, 2001). The *in vitro* biological activity of the alcoholic plant extracts was assessed on the basis of the hyphal radial growth rate of filamentous fungi and the growth rate of yeasts in the presence and absence of the plant extract. Growth of filamentous fungi was inhibited 40–80% in the presence of 0.8 mg of dry extract/ml of culture medium. The effect on yeast growth was evaluated by the agar well diffusion assay and paper disc diffusion assay (Camm *et al.*, 1975; Cole, 1994; Torres *et al.*, 1998) and MIC was determined by broth dilution test. MICs against *S. carlsbergensis* and *Rhodotorula* spp. determined by dilution tests were 400 and 200 μg of dry extract/ml, respectively. Data indicated that the extracts of *Z. punctata* have a considerable *in vitro* antifungal activity against all filamentous fungi and yeasts. It is worth noting that some members of the *Aspergillus* and *Fusarium* genera are well-known producers of aflatoxins. These secondary metabolites are potent carcinogens, hepatotoxins, teratogens and inmunosuppresive compounds (Ciegler, 1975). *F. oxysporum* produces phytotoxic fusaric acid and lycomarasmin (Ueno *et al.*, 1977). *A. niger* produces potent mycotoxins on foodstuffs and is the most prevalent fungus affecting maize. These fungi represent threats not only to the health of crops, but also to animals and humans ingesting contaminated feeds and foods.

An ethanolic extract of aerial parts of *Z. punctata* was active toward fungal pathogens isolated from soybean carpels and seeds: *Phomopsis longicolla, Alternaria alternata, Fusarium equiseti* and *Colletotrichum truncatum;* one isolate of *Sclerotium bataticola* obtained from the stem and four isolates of *Fusarium graminearum* were also evaluated (Svetaz *et al.*, 2004). Fungi were characterized by the morphology of their colonies, fruiting bodies and spores. The application of different techniques of fractionation with solvents (n-hexane, $CHCl_3$ and n-BuOH) allowed the separation of three extracts. The $CHCl_3$ fraction was chromatographed on Silica Gel 60H eluted with different solvents. Two known chalcones, 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxy-chalcone, and the flavanone 7-hydroxyflavanone were purified together with two new components, 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate and 1-methyl-3-(3′,4′-hydroxyphenyl)-propyl caffeate. Antifungal evaluation of the extracts and purified substances were performed with agar dilution assays (Mitscher *et al.*, 1972; Zacchino *et al.*, 1999). MIC (defined as the lowest concentration of extract or pure compound showing no visible fungal growth) was determined for each extract and for the purified compounds and compared with antifungals of commercial use (strobirulines pyraclostrobin, azoxystrobin, carbendazin and ketoconazole). Among the isolated substances chalcones have a potent antifungal action; the flavones and one of the new compounds (1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate) have a moderate activity; and 1-methyl-3-(3′,4′-hydroxyphenyl)-propyl caffeate did not show significant activity up to a value of 50 μg of compound/ml. Taking into account that extracts and pure compounds with MIC values >1000 or >50 μg/ml, respectively, were considered inactive (Svetaz *et al.*, 2004), it is interesting to note that four of the five compounds isolated from the chloroform extract displayed good activity (MIC ≤6.25 μg/ml) against *P. longicolla* growth, the fungus that produces serious seed decay

associated with pod and stem blight, and against *C. truncatum* growth, an antracnose-producing fungus (Henning, 1987; Piolo *et al.*, 2000). Otherwise the chalcones showed high activity against *C. truncatum* (MIC = 6.25 μg/ml). Consequently, leaves and twigs of *Z. punctata* contain antifungals against fungi isolated from soybean plants cultivated in different regions of Argentina.

Successive extractions of fruits, aerial parts and exudates of *Z. punctata* (Svetaz *et al.*, 2007) with non-polar solvents, under reflux, allowed the separation of two main fractions (petroleun ether and dichloromethane) with moderate antifungal activity against the yeasts *Candida albicans, Saccharomyces cerevisiae* and *Cryptococcus neoformance* (MIC: 62.5–250 μg/ml), and a strong antifungal activity against the dermatophytes *Microsporum gypseum, Trichophyton rubrum* and *Trichophyton mentagrophytes* (MICs: 8–16 μg/ml), supporting the possible use of this plant in pharmacological applications. Fractionation of the organic extracts demonstrated that 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcones are the main compounds responsible for the antifungal activity. Moreover, with the purpose of investigating their possible use in clinical applications, the active substances were assayed on clinical isolates from immunocompromised, infected patients.

The values of MIC_{80}, MIC_{50} and minimum fungicidal concentration (MFC) of both chalcones were analysed in an extended panel of clinical isolates in a scheme for three-dimensional steady flow with a second-order accuracy for the most sensitive fungi and also comprised a series of targeted assays. The results suggested that 2′,4′-dihydroxychalcone is a fungicide and does not disrupt the fungal membrane up to 4 × MFC or act on the cell wall. Consequently, chalcones seem to have a different action mechanism than polyene and azole existing drugs because both chalcones are fungicidal and not fungistatic like azoles (Ablordeppey *et al.*, 1999), and 2′,4′-dihydroxychalcone did not disrupt membranes as amphotericin B does (Carson *et al.*, 2002). Furthermore, 2′,4′-dihydroxychalcone seems not to act by inhibition of the growth of fungal cell walls (López *et al.*, 2001). Consequently, *Z. punctata* would be considered as a source of antifungals against skin-infecting fungi.

12.4.3 Genotoxic and anti-genotoxic activities

The possible genotoxic and anti-genotoxic effect of an ethanolic extract of *Z. punctata* and 2′,4′-dihydroxychalcone was evaluated (Zampini *et al.*, 2008). Their toxicity was assayed with the lethality test of *Artemia salina* (Finney, 1971) in order to use sublethal quantities to study cell damage in the experiments of genotoxicity. The comet assay (Moretti *et al.*, 2002) was applied for the analysis of DNA damage because it provides a direct determination of DNA single- and double-strand breaks in individual cells. This test was selected because it was applied for the *in vivo* and *in vitro* assays with several cell lines (CHO, V79, HepG2, among others) (Valentin-Severin *et al.*, 2003). The HepG2 cells present an endogenous bioactivation capacity, retain many of the morphological characteristics of liver parenchymal cells, and contain phase I and phase II drug-metabolizing enzymes (factor S9) that play an essential role in the activation/detoxification of pro-mutagens/pro-carcinogens, the latter being the major advantage of HepG2 cells for their use in mutagenicity/anti-mutagenicity studies (Knasmüller *et al.*, 1998). Controls of cell viability were performed before the assays of genotoxicity/anti-genotoxicity. The effect of the co-treatment of HepG2 cells with a direct genotoxic compound (4-nitroquinoline-N-oxyde) and *Z. punctata* extract or 2′,4′-dihydroxychalcone decreased the DNA cell damage. The pre-treatment of HepG2 cells with *Z. punctata* extract or 2′,4′-dihydroxychalcone and incubation with an indirect mutagen (benzo[a]pyrene) significantly decreased the DNA damage. Consequently, the results suggest that the alcoholic extract of *Z. punctata,* as well as one of its components, is not genotoxic. Anti-genotoxic activity was demonstrated in the chosen experimental conditions, though in this

stage of the studies is difficult to assure that *Z. punctata* and the chalcone are anti-carcinogens.

12.4.4 Cytoprotective effect

The pharmacological effect of aerial parts of *Z. punctata* extracts (infusion and acetone extract) and 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone on the gastrointestinal tract of mice and rats was evaluated through the study of the induction of gastric lesions (Ortega *et al.*, 2003). Oral administration of absolute ethanol was used as necrotizing agent (Robert *et al.*, 1979) to induce gastric lessions. The degree of erosion in the glandular part of the stomach was analysed (Guardia *et al.*, 1994). Ethanol produced gastric ulcers in all the animals treated. Pretreatment with *Z. punctata* infusion (10% at a dose of 50 ml/kg, orally), acetone extract (250 mg/kg, orally) and the isolated compounds (100 mg/kg in 0.4% carboxymethyl cellulose, orally) protected against gastric ulceration. Another important property of these products is the delay in the transit by the small intestine produced by the ingestion of castor oil as a cathartic agent, suggesting their use as an antidiarrhoeal substance. The data available indicate that extracts of *Z. punctata* and its purified compounds can be used to reduce the intestinal transit in rats and mice and as protective agents against ethanol-induced stomach ulceration in rats. The action of melatonin on the cytoprotective effect of chalcones from *Z. punctata* was assayed on ethanol-induced gastroduodenal injury in rats (de la Rocha *et al.*, 2003). In previous studies it was suggested that one of the mechanisms responsible for ethanol-induced gastroduodenal damage is the generation of free radicals (Pihan *et al.*, 1987; Szelenyi and Brune, 1988). Compounds with free-radical scavenging properties, such as thiourea, dimethylsulfoxide and sulfydryl-containing substances, significantly reduce ethanol toxicity to gastric mucosa. Also, oxygen-derived free radicals have been shown to participate in reperfusion damage both in the intestine and stomach leading to lesions that morphologically resemble those induced by ethanol (Parks *et al.*, 1983; Itoh and Guth, 1985). Recently, the pineal hormone melatonin was shown to scavenge both the hydroxyl and peroxyl radicals (Tan *et al.*, 1993; Pieri *et al.*, 1994; Poeggeler *et al.*, 1995). Both *in vitro* and *in vivo* studies have demonstrated the antioxidant properties of melatonin (Hardeland *et al.*, 1995; Reiter, 1995; Reiter *et al.*, 1995).

The intragastric administration of 2′,4′-dihydroxychalcone or 2′,4′-dihydroxy-3′-methoxychalcone (100 mg/kg, orally) inhibited both gastric and duodenal lesions induced by ethanol in rats in different degrees, but pre-treatment with melatonin (10 mg/kg, orally) increased the cytoprotective effect. Hydroxychalcones, with an ample distribution in the plant kingdom, prevent gastric mucosal lesion formation induced by oral administration of severe necrotizing agents, such as 60% ethanol in 150 mM HCl and 0.2N NaOH, suggesting that these compounds possess a potent cytoprotective property on the gastric mucosa (Yamamoto *et al.*, 1992). The mechanisms responsible for the apparent additive action of the chalcones and melatonin need to be explained. These effects could be due, in part, to the radical scavenging activity of the melatonin (Reiter *et al.*, 1995).

12.4.5 Antioxidative activity and DNA protection

Living cells are permanently exposed to potentially damaging free radicals of intracellular origin, such as those arising from normal cellular metabolism, or extracellular, originating as consequence, for instance, of exposure to ultraviolet or ionizing radiations. Of special interest are the reactive oxygen species (ROS), including the highly reactive hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet -}$), and non-radical hydrogen peroxide (H_2O_2). The cellular targets for oxidative modification by ROS include DNA, lipids and proteins (Mantena *et al.*, 2008). Consequently, the ingestion of antioxidants

is an essential need in body systems. The use of plants in traditional medicine is widespread all over the world. This knowledge is still a source for the development of novel pharmacological agents. The preservative effect of many plant species and herbs suggests the presence of antioxidants and anti-genotoxic constituents in their tissues (Hirasa and Takemasa, 1998).

We investigated the oxidative DNA damage-protecting activity and antioxidant potential of *Z. punctata* ethanolic extract (aerial parts) as well as of 2′,4′-dihydroxychalcone (DHC) and 2′,4′-dihydroxy-3′-methoxy chalcone (DHMC) employing a variety of *in vitro* methods (Vattuone *et al.*, 2008). The protective effect of different quantities of the extract from *Z. punctata* (and the isolated constituents) on the breakdown of supercoiled DNA (pBR 322 was used) by the action of the free radical $HO^{\bullet}$ generated by the UV irradiation (8000 μW/cm, 5 min) on H_2O_2 was analysed by electrophoresis in agarose horizontal slab gels stained with ethidium bromide. The electrophoretic pattern showed two bands on the agarose gel electrophoresis: the faster one corresponded to the native supercoiled circular DNA and the slower moving band (faint) was the open circular form in the control. This band is intensified by exposure of the pBR 322 to UV in the presence of H_2O_2, indicating that $HO^{\bullet}$ generated from UV-photolysis of H_2O_2 produced DNA strand scission. Moreover, this band diminished when the UV irradiation of H_2O_2 was made in the presence of DHC or DHMC, demonstrating the protective effect of DHC and DHMC on DNA. Although both O_2^- and H_2O_2 are potentially cytotoxic, most of the oxidative damage in biological systems is caused by $HO^{\bullet}$, which is generated by the reaction between O_2^- and H_2O_2 in the presence of metal ions (Guitteridge, 1984). The free-radical scavenging and antioxidant capacity of the same samples was assessed with *in vitro* (cell free) tests for their simplicity and sometimes reasonable cost. The stable 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$; Kato *et al.*, 1988) and the 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation (Re *et al.*, 1999) were used. In the two assays, DHC was more effective (percentage inhibition) than DHMC and the ethanol extract with both scavengers. There was a dose-dependent response in both methods. They were also effective in superoxide scavenging assay (Nishikimi *et al.*, 1972) in a concentration-dependent manner. In general, the free-radical scavenging activity is an accepted mechanism of antioxidants to inhibit peroxidation. Lipoperoxidation was measured in the absence and presence of a stress agent (5 μmol/l $FeCl_3$). The oxidized lipids were quantified measuring the production of thiobarbituric acid-reactive substances (TBARS) (Ohkawa *et al.*, 1979). In all instances, DHC was more efficient than DHMC and the ethanol extract.

12.5 Conclusions

Zuccagnia punctata Cav. has promising potential in the treatment of conditions such as diarrhoeal gastroenteritis, and as an antifungal, antibacterial, anti-inflammatory, cytoprotective, antioxidant and anticancer agent, among others (Anto *et al.*, 1995; Edenharder and Tang, 1997; López *et al.*, 2001; Quiroga *et al.*, 2001; De la Rocha *et al.*, 2003; Uchiumi *et al.*, 2003; Zampini *et al.*, 2005; Vattuone *et al.*, 2008). It is worth taking into account that *Z. punctata* exhibits antioxidant and anti-inflammatory effects because oxidative injury underlines many of these diseases. The diverse pharmacological activities of *Z. punctata* extracts and the isolated phytochemicals have, however, only been assayed *in vitro* or using laboratory animals, and the obtained results are not necessarily portable to humans. On the grounds of the low toxicity of the extracts and derived phytochemicals and their use as a source of medicinal agents (mainly leaves and twigs), backed by proven activity of both the traditional formulations (infusions, decoctions and tinctures) and their derived phytochemicals (chalcones, flavanones,

flavones and phenyl caffeates), further research, clinical trials and product development can only strengthen the case for *Z. punctata* as an important part of our diversity and for sustainable use for generations to come.

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13 Insecticidal Activity of a South American Plant: *Hybanthus parviflorus* (Violaceae)

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13.1 Introduction

Violaceae is a worldwide family from temperate regions comprising 22 genera and 900 species that are widely distributed and are generally perennial herbaceous plants (Hoffmann *et al*., 1992). The distribution of this family is centred in North and South America with at least 12 genera and one genus in Central America. In Argentina this family is represented by three genera: *Viola*, with 30 to 40 species, *Anchietea*, with one species, and *Hybanthus* with 16. The genera growing in this country are well differentiated from one another in their habitats as well as in their distribution. Indigenous violets growing in Argentina such as *Hybanthus* and *Anchietea* never co-exist in the same area. The botanical features of this family have been described by Cabrera (1965) and Cabrera and Zardini (1979). Sparre (1950) has provided a brief key on the most relevant differences of the three Argentine genera: *Hybanthus, Anchietea* and *Viola.*

13.1.1 The genus *Hybanthus*

The native species of the genus *Hybanthus* in Argentina are: *Hybanthus albus* (A. St.-Hil.) Baill.; *Hybanthus atropurpureus* (A. St.-Hil.) Taub.; *Hybanthus bicolor* (A. St.-Hil.) Baill.; *Hybanthus bigibbosus* (A. St.-Hil.) Hassl.; *Hybanthus calceolaria* (L.) Schulze-Menz; *Hybanthus circaeoides* (Kunth) Baill.; *Hybanthus communis* (A. St. Hil.) Taub.; *Hybanthus graminifolius* (Chodat) Schulze-Menz; *Hybanthus hasslerianus* (Chodat) Hassl.; *Hybanthus hieronymi* (Griseb.) Hassl.; *Hybanthus longistylus* Schulze-Menz; *Hybanthus paraguariensis* (Chodat) Schulze-Menz; *Hybanthus parviflorus* (Mutis ex L.f.) Baill.; *Hybanthus serratus* (Phil.) Hassl.; *Hybanthus velutinus* Schulze-Menz (Zuloaga and Morrone, 1999). *Hybanthus ipecacuana* (L.) Baill. is cited in '*Medicinal Plants of Argentina*' (Toursarkissian,1980). Also *Hybanthus leucopogon* Sparre is an endemic species (Zuloaga and Morrone, 1999).

13.1.2 *Hybanthus parviflorus*

Hybanthus parviflorus (Mutis ex L.f.) Baill is shown in Fig. 13.1. Synonymy: *Calceolaria banjii* Rusby, *Hybanthus glutinosus* (Vent.) Taub., *Hybanthus parviflorus* (Mutis ex L.f.) Baill. f. *membranaceus* Schulze-Menz, *Hybanthus parviflorus* (Mutis ex L.f.) Baill. var. *argentinensis* Sparre, *Hybanthus parviflorus* (Mutis ex L.f.) Baill. var. *glutinosus* (Vent.) Hassl., *Hybanthus*

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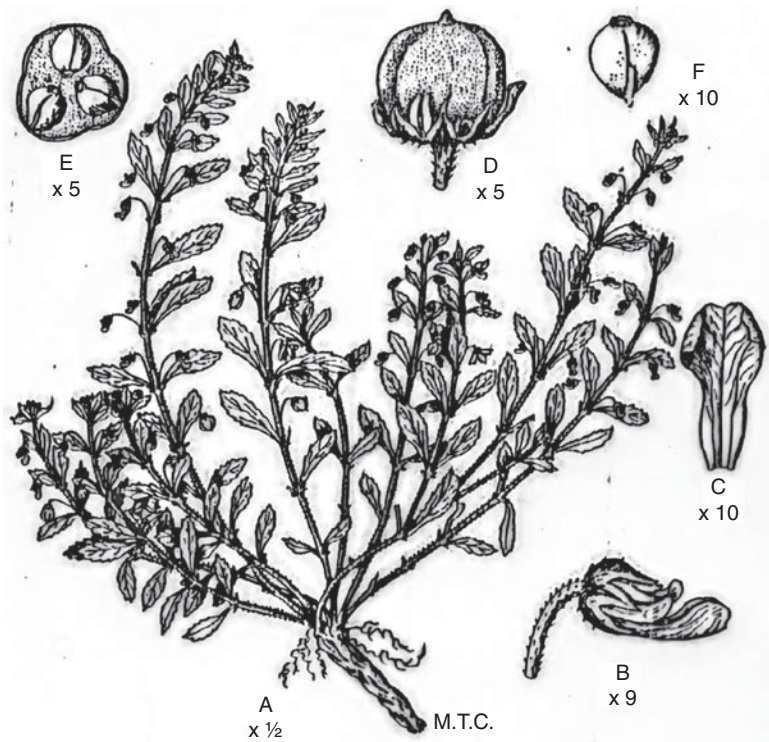

— *Hybanthus parviflorus* (Mut.) Baill.: A, planta en flor; B, flor; C, pétalo inferior; D, fruto; E, sección del fruto; F, semilla.

Fig. 13.1. *Hybanthus parviflorus* (Cabrera, 1965).

parviflorus (Mutis ex L.f.) Baill. var. *latifolius* (Eichler) Hassl., *Hybanthus parviflorus* (Mutis ex L.f.) Baill. var. *typicus* Hassl. Published with an invalid name: *Ionidium glutinosum* Vent., *Ionidium parviflorum* (Mutis ex L.f.) Vent., *Viola parviflora* Mutis ex L.f. (Zuloaga and Morrone, 1999).

H. parviflorus is a minor shrub that grows at 0–1000 m above sea level. It is an annual branchy erect plant no more than 50 cm high. This plant has opposite leaves that are shortly peciolated and serrated. It has pubescent stems and small solitary flowers that are white in colour, sometimes having a purple shade, located in the axil of the upper leaves. The fruit is a globular capsule (Cabrera, 1965). This plant blossoms and fructifies in autumn: April, May (Marzocca, 1997).

In Argentina, *H. parviflorus* grows in the provinces of Buenos Aires, Catamarca, Chaco, Córdoba, Corrientes, Entre Ríos, Formosa, Jujuy, La Pampa, La Rioja, Misiones, Río Negro, Salta, Santa Fe, San Luis and Tucumán. It is also found in the neighbouring countries of Bolivia, Brazil, Chile, Paraguay and Uruguay (Zuloaga and Morrone, 1999). *H. parviflorus* is a species that has adapted to grow in undisturbed soils. It is a weed of annual crops and is mainly found in fallows and in soybean fields under direct sowing. It also grows in stubble, wastelands, on walls and in rubble (Marzocca, 1997). Some species that are considered to be weeds, such as *H. parviflorus*, that grow during the fallows can make a significant contribution mainly during the initial stages of the implementation of an agricultural system (Papa, 2000).

H. parviflorus is a plant species that has generated biotypes resistant to the herbicides commonly employed for the control of weeds in fallows and during the cultivation cycle, such as glyphosphate and

2,4-dichlorophenoxyacetic acid (2,4-D). Because of this, *H. parviflorus* is cited among those plants belonging to the category of weeds displaying different degrees of resistance to glyphosphate (Marzocca 1993; Faccini, 2000; Pengue, 2001).

13.1.3 Ethnomedical uses

H. parviflorus is a species known by many popular names in the folk medicine of the countries where it grows:

- In Colombia it is known as 'teatina' or 'chuchunchullo'. Its roots are used in the form of decoction or as infusion as a mild laxative, emetic, to cure amoebic dysentery, the liver hypertrophy and, in general, chronic diarrhoea (García Barriga, 1992).
- In Chile it is named 'maitencillo'. Its roots are employed as a substitute of ipecacuanha because it seems to have the same medicinal properties. People living in the countryside employ it as emetic and purgative and it is admininstered as a powder or as an infusion of the roots. The decoction of the leaves mixed with the other aromatic plants is used to alleviate neuralgia, mainly those of rheumatic origin (Murillo, 1889; Pizarro, 1966).
- In Peru it is known as 'pachaga pichinya' and is used as an emetic agent (Soukup, 1986; Rutter, 1990).
- In Uruguay it is named 'maitencillo', 'maitecillo' or 'maytencillo'. With the exception of the roots, the infusion of the whole plant in a proportion of 15 g/l of water is administered in cases of rheumatism and neuralgia. At low doses, the roots have emetic and purgative properties, whereas at high doses it may cause gastroenteritis and may even cause death (Lombardo, 1979).
- In Argentina it is known as 'violetilla'. Its roots are also employed as emetic and laxative (Domínguez, 1903; Toursarkissian, 1980; Marzocca, 1997).

13.1.4 Chemical composition

Chemical studies indicate the presence of an alkaloid called violine in its roots that has similar properties to emetine. For this reason, the roots of *H. parviflorus* can be used as a substitute for the roots of ipecacuanha (Dominguez, 1903; Mateu Amengual, 1980; García Barriga, 1992).

The flavonoids quercetin, kaempferol, luteolin and apigenin have also been detected in the leaves (Harborne and Williams, 1975; Saxena, 1975; Mateu Amengual *et al.*, 1981).

13.2 Biological Activities of *H. parviflorus*

To date, no biological activities of the species *H. parviflorus* have been detected. Taking into account the presence of cyclotides in the family Violaceae, *H. parviflorus* has been selected to assess its insecticide activity. Cyclotides are known to have many biological activities, but the function of such molecules in plants has not yet been elucidated. It is speculated, however, that these compounds play a role in defence mechanisms (Jennings *et al.*, 2005).They are found in considerable amounts in the species that contain them (>1 g/kg in the leaves) and are rich in cysteine (Craik *et al.*, 1999).

These compounds have utero activity (Gran, 1973a), are microbicidal (Tam *et al.*, 1999), insecticidal (Jennings *et al.*, 2001, 2005; Gruber *et al.*, 2007; Barbeta *et al.*, 2008; Wang *et al.*, 2009), anthelmintic (Colgrave *et al.*, 2009, 2008), anti-HIV (Gustafson *et al.*, 1994, 2000, 2004; Bokesch *et al.*, 2001; Ireland *et al.*, 2008; Wang *et al.*, 2008), can inhibit the binding of neurotensin to cell membranes (Witherup *et al.*, 1994), can inhibit trypsin (Hernández *et al.*, 2000), are haemolytic (Claeson *et al.*, 1998; Göransson *et al.,* 1999; Chen *et al.*, 2006), anticancer (Göransson *et al.*, 2004), cytotoxic (Lindholm *et al.*, 2002; Svangård *et al.,* 2004; Herrmann *et al.,* 2006, 2008) and cardiotoxic (Gran, 1973b). All their biological activities assessed in mammalian cellular systems seem to be related to their interaction with membranes, a common feature of the defence molecules of plants (Felizmenio-Quimio *et al.*, 2001). A role as an antimicrobial and insecticide molecule of plants has been suggested (Jennings *et al.*, 2001).

The cyclotides found in plants are macrocyclic peptides, 28–37 amino acids long and have a cyclic backbone (the name deriving from cyclopeptides: cyclotides), that is, a continuous cycle of peptidic bonds (amide bonds) forming the backbone. They contain six cysteine residues (Cys) that form three disulfide bridges that stabilize the molecule (Craik *et al.*, 1999). Because of their well-defined structure and potent biological activities, these molecules can be regarded as miniproteins (Felizmenio-Quimio *et al.*, 2001). Among these miniproteins, Kalata B1, the circulins, cyclopsychotride and several peptides from *Viola arvensis* can be found. The physical properties of cyclotides, which include resistance to thermal and enzymatic breakdown (Colgrave and Craik, 2004; Ireland *et al.*, 2006), can be attributed to the cyclic backbone and disulfide bridges that, by means of the formation of a cystine knot, provide the molecule with a great stability (Craik *et al.*, 2002).

Cyclotides are aligned according to the six conserved Cys residues (named I to VI). The backbone loops of the cyclotide corresponding to the region located between the Cys residues are numbered from 1 to 6. The amino-acid chains responsible for the biological activities of cyclotides are located in these loops. The outstanding stability of the cystine knot structure makes it an excellent framework on which to add a wide range of biological activities (Craik, 2001). Most of the cyclotides described so far in the plants belonging to the Violaceae and Rubiaceae families (Trabi and Craik, 2004; Trabi *et al.*, 2004; Simonsen *et al.*, 2005; Gruber *et al.*, 2008) have sequences that allow classifying them into two subgroups or main families named 'bracelet' and Moëbius cyclotides. As established by Craik *et al.* (2002), this nomenclature is based upon the presence or absence of a *cis*-proline residue in loop 5, which introduces a twist into the circular backbone of the peptide. Thus, those cyclotides bearing such twist are named Moëbius cyclotides (Rosengren *et al.*, 2003; Daly *et al.*, 2006).

Even though cyclotides have been found in roots, stems and leaves of *Oldenlandia affinis*, these molecules are mostly found in young leaves. Therefore, the possibility that these compounds could function in defence mechanisms against plant pathogens was studied (Alexander, 2001). To this end, studies were carried out on the basis of the ingestion of diets containing 0.15% w/v of Kalata B1 by larvae of *Helicoverpa punctigera* (Lepidoptera), a moth that affects the harvests. Kalata B1 was able to inhibit the growth of these larvae, hampering the development of the first stage instar. It was not clear, however, whether this alteration in the growth of larvae was due to a toxic effect or to an antialimentary action that led to death from starvation (Jennings *et al.*, 2001).

It is known that small cysteine-rich peptides present in plants have protease or α-amylase inhibitory activities that can delay the growth of insects by blocking the digestion of proteins or starch. Nevertheless, Kalata B1 and B2 are thought to have a different mechanism of action, because they did not have any effect on the trypsins, chemotrypsins or α-amylase isolated from the gut of *Helicoverpa* sp. (Baillie Gerritsen, 2002). Furthermore, Kalata peptides possess haemolytic activity. The latter observation raises the hypothesis that the insecticide activity is the result of damage to the gut membranes of the insect (Jennings *et al.*, 2001). The insecticide activity is a remarkable property of cyclotides from both ecological and environmental points of view, having technological and economical–commercial applications. Trabi and Craik (2002) have stated the importance of studying the insecticide activity of cyclotides.

13.3 The Preparation and Purification of Extracts

13.3.1 Extraction of the plant material

Hybanthus parviflorus (*syn. Ionidium glutinosum* Ventetat, *Viola parvifolia* Roemer and Shultes; Ballard and Jorgensen, 1997), family Violaceae, was collected, in part, at the intersection of the Number 12 National Road and the Feliciano stream, La Paz Department, in the Province of Entre Ríos,

Argentina in October 1998. The plant was identified by Dr Juan de Dios Muñoz, keeping a voucher specimen – Muñoz 1514 (ERA) – in the Herbarium of the School of Agricultural Sciences, National University of Entre Ríos, Paraná City, Argentina. Specimens were also collected in Cerro Azul, Experimental Station of the National Institute of Agricultural Technology (INTA), J. Urdampilleta, L. N. Alem Department, in the Province of Misiones, Argentina, on 14 April 2002. This vegetal material was identified by Dr Aníbal Amat, and a voucher specimen is kept in the Herbarium of the Pharmacy Department of the School of Exact, Chemical and Natural Sciences (MNEF 3980).

The method employed for obtaining and purifying the extracts was described by Claeson *et al.* (1998) with the aim of obtaining peptide-enriched extracts. The vegetal material was dried under sunlight and in a forced air oven at a temperature below 40°C, to preserve it and to avoid any enzymatic degradation of the compounds present in it. According to Claeson *et al.* (1998), there are reports on plant peptides that contain several disulfide bridges, rendering them stable to heat and even to solution in boiling water. The dried and ground aerial parts of *H. parviflorus* (30.9 g) were extracted by maceration with CH_2Cl_2 (300 ml) for 1 h under continuous shaking. This procedure was repeated seven times, changing the solvent each time.

Unlike other lipophilic substances, such as chlorophylls, lipids and other substances of lower molecular weight (terpenoids and phenylpropanoids), polypeptides are not soluble in CH_2Cl_2. The insecticide activity was assessed on the dried CH_2Cl_2 extract. The plant residue was then macerated in 50% v/v ethanol in water. The latter solution is a better solvent than pure water or the alcohol to solubilize the polypeptides. Moreover, with this solvent the extraction of most polysaccharides and enzymes is avoided, and microbial growth is inhibited as well.

13.3.2 Extract purification

The ethanolic extract was acidified with 2% AcOH and eluted through a polyamide column in order to remove the tannins that bind to the polyamide with high affinity and in an irreversible fashion. This methodology has proved to be efficient in removing the tannins that were not desired for the studies. The peptides are not therefore retained in the column matrix. The column was eluted with 2% AcOH with a subsequent rinsing with 50% EtOH/2% AcOH to elute those peptides that are insoluble in 2% AcOH (Broussalis *et al.*, 2001).

The remains of the extraction with 50% v/v EtOH were macerated in 25% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). The extraction of the remaining peptides of the ethanolic extraction was assayed with the same solvent mixture as that employed in the high-performance liquid chromatography (HPLC) fractionation. The ACN extract obtained by this methodology was eluted in a polyamide column in order to eliminate any residual tannins. The same mixture that was employed in the extraction step (25% ACN/0.1% TFA) was employed as an elution solvent.

The next procedure, liquid–liquid partition BuOH/H_2O, was aimed at obtaining the cyclotides. The partition between water and BuOH of the tannin-free 50% EtOH extracts and tannin-free ACN was performed, taking into account the remarkable hydrophobic nature of the cyclotides and their solubility in BuOH. A sample of the lyophilized ethanolic extract (42.0 mg) was solubilized in Milli Q water (10 ml) and partitioned three times with 10 ml of n-BuOH. The organic and aqueous phases were separated by centrifugation (10,000 rpm, 20 min) and subsequent settling. The butanolic phase was dried under reduced pressure, at a temperature lower than 40°C. This procedure yielded a sample of 8.5 mg. The aqueous phase was dried in a Speed Vac concentrator with a refrigerated trap, obtaining 12.6 mg of dried material. Both the butanolic and aqueous fractions were subjected to HPLC (Broussalis *et al.*, 2001).

The purification process developed for this work is simpler than the methodologies previously described, such as gel filtration on Sephadex G10 with subsequent extraction on solid phase (Claeson *et al.*, 1998).

The cyclotides present in the butanolic fraction can be readily subjected to HPLC. Therefore, BuOH/H_2O partitions of the tannin-free 50% EtOH and ACN extracts were carried out, and the butanolic and aqueous fractions of each extract were analysed by HPLC with diode-array detection (DAD). Upon analysing the fraction elution pattern using an UV detector with a photodiode array, the butanolic fraction was chosen to continue with the isolation of the cyclotides because of its greater peptidic content.

Owing to the cyclic nature of the backbone and the presence of the cystine knot, which generates a tension in the molecule, the hydrophobic amino acids are forced to be exposed on the surface of the molecule instead of locating themselves inside the peptide structure (Craik *et al.*, 2001). This feature would explain the behaviour of the cyclotides during their isolation and purification, such as the longer retention times (rt), greater than 20 min, observed in reversed-phase (RP)-HPLC and the capacity to solubilize in the organic phase during the BuOH/H_2O partition.

13.3.3 Fractionation and analysis of the peptides

Isolation and purification of the peptide hypa A

The elucidation of the primary structure of a protein comprises the isolation, purification and subsequent determination of the amino-acid sequence of the purified protein and the sequence of the fragments obtained by chemical and enzymatic procedures. The cyclotide-enriched fractions of *H. parviflorus* (tannin-free 50% EtOH and ACN extracts and their corresponding butanolic and aqueous fractions) were analysed by HPLC with a UV detector with a photodiode array. The UV detection was performed at 200–300 nm. The UV light absorption of proteins in the range of 230–300 nm is due to the tyrosine (λ_{max} = 274 nm), tryptophane (λ_{max} = 280 nm) and phenylalanine (λ_{max} = 257 nm) aromatic rings, together with the contribution of the peptidic links, which display a strong absortion at wavelengths <230 nm. Disulfide bonds present a weak absorbance at ~250 nm. The tyrosine and tryptophan residues are those that contribute most to the UV absorption spectrum of a protein (Wetlaufer, 1962).

The RP-HPLC analysis of the tannin-free 50% EtOH and ACN extracts and their butanolic and aqueous fractions allowed the selection of the butanolic fraction corresponding to the 50% EtOH extract because of its greater peptidic content. Under these experimental conditions, the rt for these peptides was greater than 20 min.

The butanolic fraction was subjected to Superdex™ gel filtration chromatography for peptides. The mass spectrometry (MS)–matrix-assisted laser desorption/ionization (MALDI) spectrum of this fraction showed the presence of a series of compounds of which the molecular weight corresponded to the cyclotides (3120–3221 Da).

The isolation of the peptides by semi-preparative RP-HPLC was then performed. Briefly, a 21.38 µg/µl butanolic solution of the peptides was subjected to a semi-preparative C18 chromatography column. An isocratic mobile phase of 25% ACN in 0.1% TFA (20 min) was employed followed by a linear gradient to reach 60% ACN in 0.1% TFA. The fraction eluted with 25% ACN in TFA 0.1% (90 mg) was kept for flavonoid analysis. Nine fractions were prepared by elution with the linear gradient. The peptides started to elute after 20 min with the increase in the percentage of the organic modifier (ACN) and the concomitant decrease in the polarity of the mobile phase (Goranssön *et al.*, 1999).

Analysis of the UV spectra of the eluted substances revealed the presence of a complex mixture of peptides. Of the nine preparative peptidic fractions isolated by RP-HPLC, the fraction displaying a peak absorbance was selected. This fraction was subjected to analytical chromatography to assess its purity, employing a mobile phase containing ACN, TFA and *i*PrOH and obtaining 0.2 mg. The fraction purified by this procedure was analysed by MS-MALDI, displaying a single peak

corresponding to the cyclotide hypa A for which the structure could be determined.

Determination of the primary structure of the hypa A peptide

To assess the primary structure, the total amino-acid content of the cyclic peptide was determined after the hydrolysis with 6N HCl at 110°C for 24 h (Penke *et al.*, 1974). The cysteine was determined as cysteic acid following the methodology described by Moore (1963). The tyrosine (Y) was protected from the HCl action by using phenols as radical scavengers. The amino acids released during the hydrolysis were identified and quantified by C18 RP-HPLC employing ninhydrine as detection reagent.

To analyse the sequence, the peptide was reduced with DTE in a buffer containing EDTA and guanidine-HCl. The guanidine acts as a denaturing agent but the disulfide bonds are not cleaved by this agent. The treatment of the peptide with DTE reduces the cystines with the subsequent cleavage of the disulfide bonds and transforming them into cysteine residues. The addition of 4-vinylpiridine (4-VP) to the peptide solution prevents formation of new disulfide bonds and leads to the formation of the S-(β-4-piridylethyl) cysteine (PEC) derivative, which is more stable during the sequencing process and more easily detected (PEC –λ_{max}= 254 nm), and has a UV spectrum that is different from 4-VP (λ_{max} = 254 nm; Anders, 2002).

Craik *et al.* (1999) have assayed a wide range of proteases on the cyclotides, finding that the cystine knot (CCK) is resistant to enzymatic cleavage. The enzymatic digestion of the cyclotide employing proteases is therefore only possible after the removal of the cystine knot by the reduction of the disulfide bonds. Thus, the proteolytic digestion of the CCK required reduction, S-alkylation and subsequent cleavage of the cyclic backbone. The PEC derivative was desalted and isolated by gel filtration chromatography. The empirical molecular weight was 3780 Da, a similar value to the theoretical molecular weight of the derivative in which six cysteines reacted with 4-VP (3780 Da = 3143 + 6 [H] + 631 [4VP:105.14 × 6]).

It is known that the cyclization of the N terminus (e.g. due to the presence of a cyclized glutamine or glutamate) does not allow the determination of the protein sequence. For this reason the cyclic PEC derivative was treated with endoproteinase Glu-C to obtain a linear peptide. Endoproteinase Glu-C is a serine protease that cleaves the peptidic links of the C terminus bearing glutamic acid. This proteinase has a better specificity in ammonium bicarbonate pH 7.8. Because the hypa A pepide contains only one glutamic acid (E) its cyclic PEC derivative was readily digested with endoproteinase Glu-C, rendering a single linear product that needed no further treatment before sequencing.

The linear peptide obtained by this procedure was isolated by RP-HPLC. The molecular weight observed for the derivatized peptide (3798 Da) was the same as the calculated molecular weight (3798 Da = 3780 + 18). The complete sequence of this derivative was determined automatically by Edman's degradation. The N terminus Edman's method is the most frequently used procedure for the sequencing of proteins (Matsudaira, 1993). Finally, the sequence of the hypa A cyclotide was determined (Broussalis *et al.*, 2001).

Verification of the sequence of the hypa A peptide

The combination of HPLC and MS has proved to be an efficient method for the analysis of peptides and proteins. During the past years, both MS and MS/MS have become commonly used methods for the identification and characterization, including the determination of the molecular weight, of these compounds. These spectroscopic techniques give information on both the structure and the sequence of the peptide. However, some issues must be borne in mind for this methodology to be successful: the peptides and their fragments must have a suitable molecular size and charge to carry out the analysis, which is

10–15 charged amino acids at most, located at the C terminus.

Taking into account the natural abundance of basic amino acids, the latter condition is an expected result after the tryptic digestion of proteins and peptides. However, the inconvenience associated with the determination of cyclotides by MS/MS is related to the presence of the CCK, which requires the previous breakage and alkylation of the disulfide bonds and the rupture of its cyclic backbone. Due to the scarcity of positively charged amino-acid residues, the enzymatic degradation renders undesired fragments that are too long or that have an inadequate charge (Göransson *et al.*, 2003). In order to obtain fragments with a suitable size for analysis, in this work, the endoprotease Glu-C was employed along with trypsin.

To verify the amino-acid sequence by MS/MS, the cyclotide was reduced and treated with iodoacetamide, generating a series of derivatives with a mass increase of 58 Da per each reduced Cys that reacts with iodoacetamide. The cleavage with Glu-C rendered a linear peptide of 30 amino acids in length (from S1 to E30). Because this linear peptide was too long to be analysed by MS/MS it was treated with trypsin, obtaining three tryptic fragments: S1 to K18, N19 to K20 and V21 to E30. These peptides were isolated by RP-HPLC and analysed by nanospray ion trap MS/MS (35% CID). The experimental masses $[M+H]^+$ were assigned to the fragments according to Biemann's nomenclature (Biemann, 1990). The two sequenced peptides were 28–30 amino acids long.

The assignment of the **b** series (N-terminus fragments) and the **y** series (C-terminus fragments) allowed the verification of the tryptic fragment of 18 amino acids, S1 to K18. It was observed that the presence of lysine (K), which is a basic residue, confers a positive charge on the fragment.

In the case of the 10 amino acid long tryptic fragment (V21 to E30), the fragmentation of the ion 1183.3 $[M+H]^+$ allowed verification of the 10 amino acids located before the cleavage site of Glu-C: V21 to E30 (Broussalis *et al.*, 2001).

The low frequency of basic amino acids or, in this case, the complete absence of them, and the formation of clusters of basic amino acids, do not allow a precise assignation to this peptide. In this spectrum b^o is b- H_2O. In this peptide the presence of lysine (K) was not observed, whereas glutamic acid (E) was present, an acidic residue that confers a negative charge to this fragment. The remnant peptide N19–K20 was confirmed by tandem MS of the linear peptide of 30 amino acids obtained by partial digestion with trypsin (Fig. 13.2).

This novel cyclotide exhibited a great sequence homology with that of the cyclotides previously identified in the Violaceae and Rubiaceae families (Craik *et al.*, 2001). Furthermore, this cyclotide is 90% identical to the most closely related homologue, cycloviolacin O1, which has been isolated from *Viola odorata*. The disulfide bonds between the Cys residues in hypa A, C2–C17, C7–C22 and C15–C28 are identical to those reported by Craik *et al.* (1999) for cycloviolacin O1, as determined by nuclear magnetic resonance (NMR).

According to the sequence alignment, hypa A would belong to the subfamily 1, that is, the 'bracelet' cyclotides, owing to the absence of a *cis*-amide link between Trp2 and Pro3 (absence of a *cis*-Pro residue in the loop 3), a feature of the subfamily 2, the Möebius cyclotides (Craik *et al.*, 2002). It is also noteworthy that there is a difference in the net charge between both families: as a rule, the peptides belonging to the subfamily 1 have a net charge of +2, the value found for hypa A, whereas those cyclotides belonging

Fig. 13.2. The amino acid sequence of the cyclotide hypa A (Broussalis *et al.*, 2001). The cleavage site of endoproteinase Glu-C, marked with an arrow, was chosen as an arbitrary starting point of the numbering of the amino acids (trypsin cleavage sites are marked with dashed arrows).

to the subfamily 2 are neutral or slightly negative (Craik *et al.*, 1999).

To date, cyclotides have been found in the genera *Leonia* and *Viola*, belonging to Violaceae. In this work we have identified for the first time a novel cyclotide in the genus *Hybanthus*, which belongs to the same plant family. The presence of cyclotides in *Hybanthus* could therefore be regarded as a chemotaxonomic marker for this genus. More than 100 cyclotides have been identified so far, and new structures continue to be isolated and described. Because the number of compounds in this family is increasing, we have proposed that the trivial name for each compound be formed with the acronym of the Latin name or the scientific name of the plant from which they are isolated for the first time, and followed by a letter indicative of the order of appearance. Thus, the first cyclotide isolated from *H. parviflorus* has been named hypa A (Broussalis *et al.*, 2001). It is also worth noting that hypa A is the first cyclotide isolated from an Argentine vegetal species.

13.3.4 Phenolic compounds

Flavonoids and caffeoylquinic acids have been reported to have diverse biological activities that can be related to the insecticide activity of *H. parviflorus*. For this reason, the polyphenolic compounds (flavonoids and caffeoylquinic acids) present in the CH_2Cl_2 and 50% EtOH extracts of *H. parviflorus* have been studied by thin-layer chromatography (TLC) and HPLC. The TLC analysis allowed the determination of the presence of rutin – the principal compound – and chlorogenic acid in the 50% EtOH extract by means of the three chromatographic systems employed. The analysis of the UV spectra of the compounds present in CH_2Cl_2, 50 % EtOH and its butanolic fraction, subjected to HPLC-DAD, revealed the presence of other flavonoids and caffeoylquinic acids and allowed quantification of the principal flavonoid rutin. The presence of rutin and chlorogenic acid were detemined in the 50% EtOH extract by TLC (Broussalis *et al.*, 2010).

The CH_2Cl_2 and 50% EtOH extracts and the fraction eluted with 25% ACN–0.1% TFA of the tannin-free butanolic fraction of the 50% EtOH extract were analysed by HPLC employing a C18 column. The 50% EtOH extract was subjected to HPLC employing a Phenomenex™ C18 analytical column employing apigenin, luteolin, kaempferol, quercitin, quercitin-methyl-ether and rutin as reference flavonoids. The mobile phase consisted of the following solvents: A: H_2O-AcOH (98:2) and B: MeOH-AcOH (98:2). The following flavonoids were identified by comparing their rt and UV spectra with the rt and UV of the corresponding commercial standards: quercetin (rt 26.59 min), luteolin (rt 28.04 min), quercetin-3-methyl-ether (rt 28.47 min), kaempferol (rt 30.48 min) and apigenin (rt 31.32 min) and the glycoside rutin (rt 18.62 min). Quercetin-3-methyl-ether and rutin were identified for the first time in *H. parviflorus.*

Because the amount of rutin present in the 50% EtOH extract was considerably higher than the other flavonoids, its presence was quantified. The rutin content, as determined by HPLC, was 1.34 g/100 g extract (1.34%) or 0.24% when referring to dry powder (Broussalis *et al.*, 2010). In this extract the insecticide activity was tested. The pharmacological activities of the flavonoids apigenin, luteolin, kaempferol, quercitin and quercitin-3-methyl-ether and the rutin glycoside have been previously reported; however, such compounds continue to be studied in the search for novel biological activities and therapeutic applications (Havsteen, 2002; Menendez *et al.*, 2008).

The presence of caffeic and chlorogenic acids was assessed in the ethanolic extract of *H. parviflorus* by HPLC, employing reference substances. The butanolic fraction of the 50% EtOH extract eluted with 25% CAN–0.1% TFA that had been kept for the analysis of flavonoids was subjected to semi-preparative RP-HPLC employing 25% ACN–0.1% TFA as mobile phase. Upon analysing the UV spectra of this fraction, the presence of flavonoids could be demonstrated.

To analyse this fraction, the solvents A: H_2O-AcOH (98:2) and B: MeOH-AcOH (98:2) were employed as mobile phase. The injection volume was 100 µl and the flow rate 1.3 ml/min. Under these experimental conditions a triglycoside of the flavonoid rutin, named QT, was isolated.

The presence of other flavonoids was also found in the CH_2Cl_2 extract of *H. parviflorus*; one of them (rt 45.03) has also been found in the ethanolic extract. The polyphenols present in the CH_2Cl_2 extract are thought to contribute to its insecticide activity (Azcon-Bieto and Talon 1993; Hashim and Devi, 2003).

13.3.5 Steroidal and triterpenic compounds: β-sitosterol, oleanoic acid and ursolic acid

The presence of β-sitosterol in the CH_2Cl_2 extract of *H. parviflorus* was determined by TLC and gas chromatography (GC) employing a standard of β-sitosterol. The oleanoic and ursolic acids were analysed by TLC in two chromatography systems. The GC analysis of the CH_2Cl_2 extract of *H. parviflorus* employing standards of oleanoic and ursolic acid, and the co-chromatography of the extract employing the corresponding standards, allowed confirmation of the presence of such acids in the extract (Broussalis *et al.*, 2010).

A wide range of biological and pharmacological properties have been reported for β-sitosterol as well for the oleanic and ursolic acids, being of great interest for their therapeutic and industrial uses. The insecticide activity of the CH_2Cl_2 extract could be attributed or be reinforced by the anti-alimentary effect proven for the β-sitosterol and the oleanic and ursolic acids (Shukla *et al.*, 1996; Chandramu *et al.*, 2003; Mallavadhani *et al.*, 2003).

13.4 Insecticide Activity of *H. parviflorus*

Both plants and herbivorous animals have co-evolved over hundreds of years, and as a result of this evolution, plants have acquired defence mechanisms against nematodes, insects, birds and mammals (Jongsma and Bolter, 1997). It is only when herbivorous animals can adapt to these defence mechanisms that they may potentially become plagues.

The function of cyclotides as plant defence mechanisms against microorganisms and insects has been mentioned above. Besides, the anti-alimentary effect of the ursolic acid on lepidopterous insects, e.g. *Spodoptera litura* (the tobacco plague), has been reported. β-Sitosterol has also been demonstrated to have an anti-alimentary effect. Taking into account these effects, the insecticidal activity of the extracts and fractions of *H. parviflorus* were investigated. To this end, the lethal and sublethal effects of such extracts and fractions were investigated on the fruit fly (*Ceratitis capitata*). This insect (Fig. 13.3a,b), commonly known as the Mediterranean fly or fruit fly, is a worldwide plague, and in Argentina it is one of the most important plagues of fruit cultivations, especially those of citrus. This fly directly affects the fruit during the maturing process in the tree. *Ceratitis capitata* has a short life cycle, it is easy to manipulate and has a high fecundity rate, all features that make it suitable for biological assays (Bado *et al.*, 2004).

The insecticidal activity of the CH_2Cl_2, 50% EtOH, tannin-free ethanolic extracts and the butanolic and aqueous solutions as well as the ACN extract were investigated (Broussalis *et al.*, 2010). The concentrations employed were: CH_2Cl_2 extract, 1000 and 100 ppm; 50% EtOH extract, 1000 and 100 ppm; tannin-free 50% EtOH extract and its butanolic and aqueous solutions, 200 ppm each one; and ACN extract, 200 ppm. The mortality at each stage of the life cycle of the fly as well as the overall mortality was assessed. The delay in the development of the insect produced by the CH_2Cl_2 and 50 % EtOH extracts was also evaluated (Fig. 13.3c,d,e,f).

Analysis of variance and Tukey's test (Steel and Torrie, 1993) were employed as statistical methods for the analysis of the results. Dose–response curves were analysed by probit analysis (Finney, 1971).

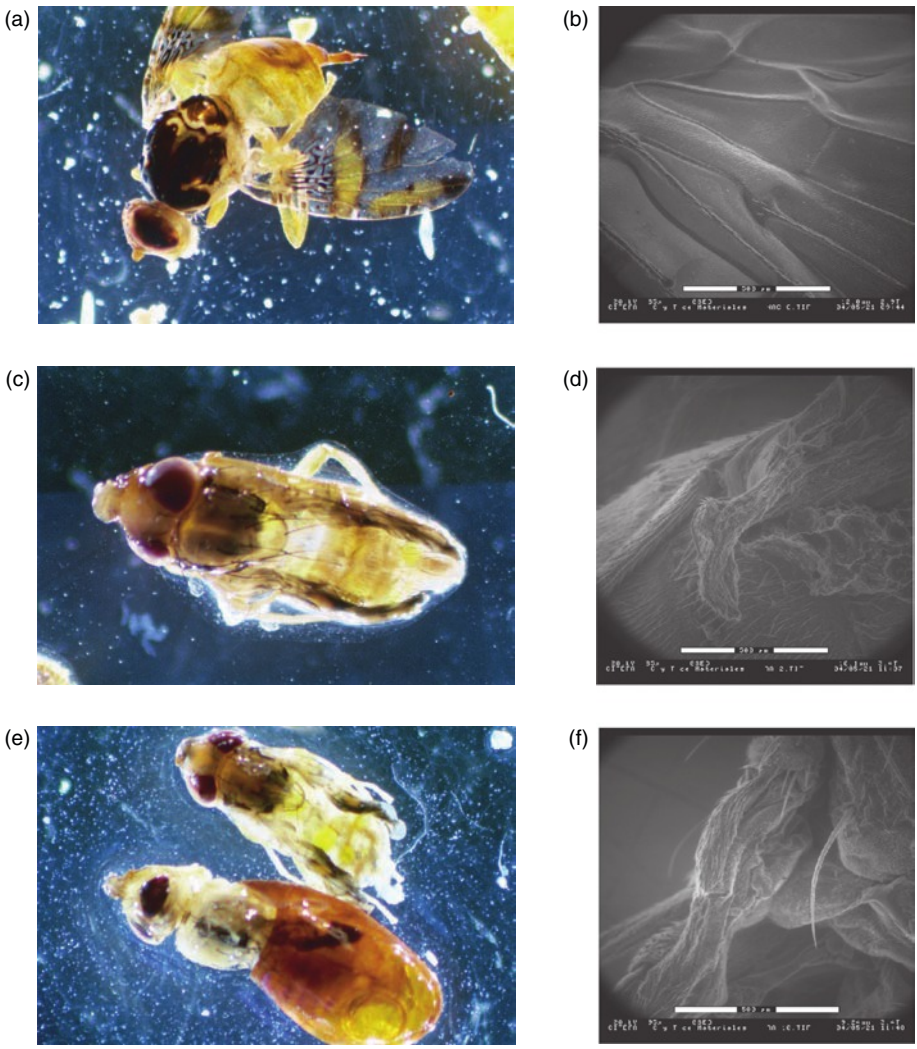

Fig. 13.3. Insecticidal activity on *Ceratitis capitata*. (a) Normal adult, adult individual size, length: 5.5 mm on average; wingspan: 10 mm. (b) Normal adult, detail of the wing, scale: 500 γm. (c) CH_2Cl_2 extract at 100 ppm, defective adult. (d) CH_2Cl_2 extract at 100 ppm, defective adult, detail of the wing and leg, scale: 500 γm. (e) 50% EtOH extract at 1000 ppm, defective adult (above) and adult with incomplete emergence (below). (f) 50% EtOH extract at 1000 ppm, detail of non-unfurled wings, scale: 500 γm. Photos a, c and e courtesy of Dr Sandra Clemente; photos b, d and f courtesy of Dr Sandra Clemente and Dr Adriana M. Broussalis. Photos b and f reprinted from Broussalis *et al.*, 2010, with permission from Elsevier.

13.5 Mortality at Each Stage of the Life Cycle of *C. capitata* and Overall Mortality

The effect of the CH_2Cl_2 and 50% EtOH extracts at 1000 and 100 ppm were evaluated on puparium formation as well as on the overall mortality. The effect of such extracts on insect mortality was also recorded as emergence or non-emergence of the adult stage. Results are shown in Table 13.1 (Broussalis *et al.*, 2010).

Table 13.1. Effect of CH_2Cl_2 and 50% EtOH extracts at 1000 and 100 ppm on puparium formation and mortality of *Ceratitis capitata*.

Extract (ppm)	Without puparium formation (%)	Adult emergence (%)	Without adult emergence (%)	Overall mortality (%)
Control	0[a]	100	0	0[a]
CH_2Cl_2 (1000)	53[b]	10	37	90[b]
CH_2Cl_2 (100)	32[b]	18	50	100[b]
50% EtOH (1000)	30[b]	15	55	100[b]
50% EtOH (100)	53[b]	10	37	95[b]

[a,b]Significant differences with respect to control (p <0.05).

13.5.1 Delay in the development of the insect

The effect of the CH_2Cl_2 and 50% EtOH extracts at 1000 and 100 ppm were evaluated on the delay in the development of the fruit fly recorded as pupariation time. Significant differences were found in the pupariation time obtained with the ethanolic extract at both concentrations employed (Table 13.2) (Broussalis *et al.*, 2010).

13.5.2 Pre-pupariation mortality

The CH_2Cl_2 and the 50% EtOH extracts induced mortality at 100 and 1000 ppm, but only the 50% EtOH extract induced delays in the development time. The effect of the 50% EtOH and the tannin-free 50% EtOH extracts, the butanolic and aqueous solutions and the ACN extract were therefore evaluated on the pre-pupariation mortality of the fruit fly. Each solution was assayed at 20 ppm (Table 13.3) (Broussalis *et al.*, 2010).

The lethal effects were evaluated as mortality percentage at each developmental stage and as overall mortality. To this end, the effects of the CH_2Cl_2 and 50% EtOH extracts at 1000 and 100 ppm were evaluated on the puparium formation and the mortality of the fly. The mortality of the adult stage was also evaluated and recorded as emergence or non-emergence percentages (Fig. 13.4).

As for the overall mortality, both extracts induced a high mortality percentage, even at low concentrations (CH_2Cl_2, 100 ppm: 100%; 50% EtOH, 100 ppm: 95%), and as a consequence, a low emergence of adults percentage was observed. Results also showed that at 100 ppm the 50% EtOH extract inhibited puparium formation 53%, whereas at 1000 ppm this was 30%. On the contrary, the CH_2Cl_2 extract at a low concentration (100 ppm) inhibited the puparium formation 32% and 53% for the higher concentration (1000 ppm).

For the mortality of adults, it was observed that on treatment with the 50% EtOH extract at 1000 ppm, 15% of emerging adults died thereafter (Fig. 13.3e,f), whereas at 100 ppm of the extract only 10% of the adults emerged, 5% of which survived. In the case of the CH_2Cl_2 extract, at 1000 ppm 10% of the emerging adults survived, and when this extract was used at 100 ppm 18% of the adults that emerged died thereafter (Fig. 13.3c,d).

The sublethal effects were also assessed as delays in the development induced by the CH_2Cl_2 and 50% EtOH extracts at 1000 and 100 ppm (Fig. 13.5).

The 50% EtOH extract delayed the development of the fruit fly with similar values for both concentrations. Thus, the time necessary for the pupariation of 50% (PT_{50}) of the flies treated with this extract at 1000 ppm was 11.3 days, and the PT_{50} for the flies treated with 100 ppm of the extract was 12 days. The value for untreated flies was 5.6 days.

The results presented herein demonstrate the insecticidal effects of both the CH_2Cl_2 and the 50% EtOH extracts of *H. parviflorus*. Nevertheless, delays in the development of the fruit fly were only

Table 13.2. Effect of CH_2Cl_2 and 50% EtOH extracts at 1000 and 100 ppm on delay in the development of *Ceratitis capitata*.

Extract (ppm)	Pupariation time PT_{50} (days)	Confidence interval	Significant differences
Control	5.6	5.3–5.7	a
CH_2Cl_2 (1000)*	–	–	–
CH_2Cl_2 (100)	5.6	5–6.1	a
50% EtOH (1000)	11.3	9.8–19.6	b
50% EtOH (100)	12	10.2–25	b

PT, pupariation time; *<50% of pupariated specimens; unable to calculate. a,b, significant differences with respect to control ($p < 0.05$).

Table 13.3. Effect of extracts on pre-pupariation mortality of *Ceratitis capitata*.

Extract or fraction	Mortality %	Significant differences
Control	0	a
50% EtOH	80	c
Tannin-free 50% EtOH	100	c
BuOH fraction	90	c
Aqueous fraction	65	b
CAN	58	b

a, b and c, significant differences with respect to control ($p < 0.05$).

observed upon treatment with the 50% EtOH extract. Taking into account that the 50% EtOH extract contains cyclotides and with the aim of determining whether these compounds were responsible for the insecticidal activity observed, the pre-pupariation mortality was evaluated employing the 50% EtOH extract and its corresponding purified fractions; that is, the tannin-free 50% EtOH extract and its butanolic and aqueous fractions and the ACN extract of *H. parviflorus*. Since the 50% EtOH extract was active at 1000 and 100 ppm, tannin-free and purified fractions were employed at 200 ppm (Figure 13.6).

All the extracts and fractions induced a high mortality percentage. However, the mortality induced by the butanolic fraction obtained by the purification of the tannin-free 50% EtOH extract was higher (90%) than the mortality induced by the aqueous fraction and the ACN extract. This result is in agreement with the greater proportion of cyclotides present in this fraction that display a high solubility in BuOH. The aqueous fraction (65% mortality) and ACN extract (58% mortality) contain cyclotides but in smaller quantities, as determined by HPLC-DAD.

The insecticidal activity of the 50% EtOH extract, its purified fractions and the ACN extract that contain cyclotides could be explained by the observations of Jennings *et al.* (2001) who demonstrated that the cyclotide Kalata B1 is able to inhibit the

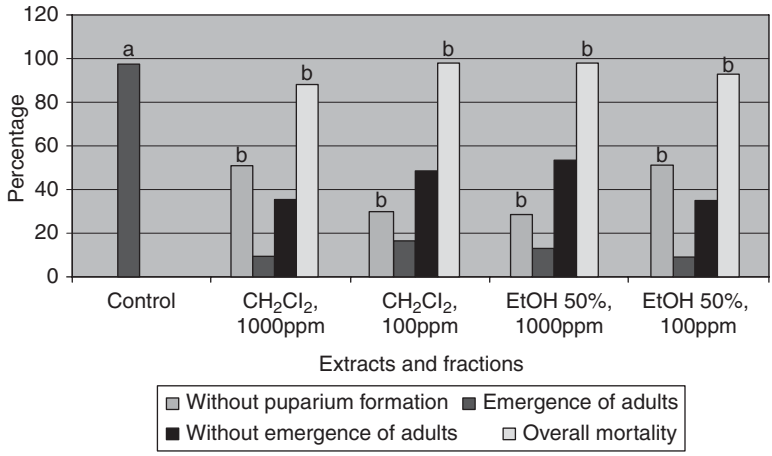

Fig. 13.4. Mortality at each developmental stage and overall mortality. a and b, significant differences with respect to control ($p < 0.05$).

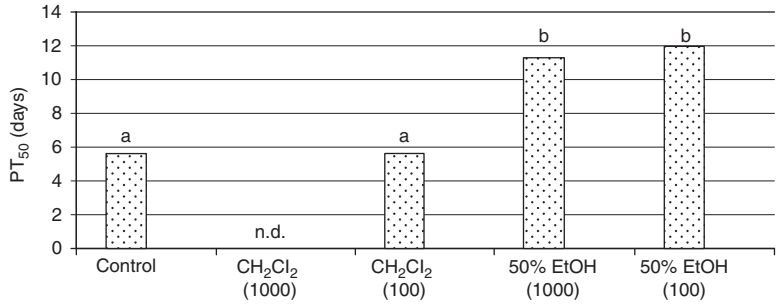

Fig. 13.5. Delays in development. PT_{50} (Pupariation time 50%): time necessary for 50% of the individuals under study to pupariate. a and b, significant differences with respect to control (p <0.05). n.d., not determined.

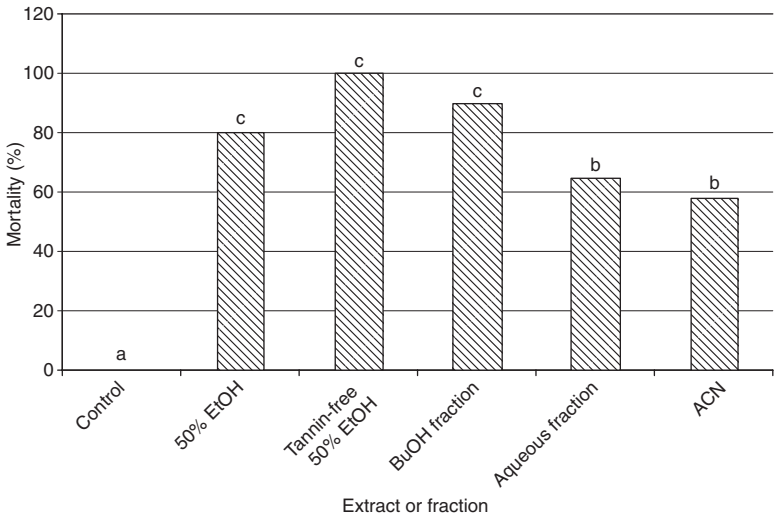

Fig. 13.6. Pre-pupariation mortality. a, b and c, significant differences with respect to control (p <0.05).

development of the moth *Helicoverpa punctifera*. These authors did not determine whether the insecticidal activity was due to a toxic effect of the cyclotide or to an antialimentary effect leading to death by starvation of the insect. Kalata B1 also presented haemolytic activity; this effect would explain the insecticidal activity by damage to the membranes of the insect's gut. It should also be borne in mind that insects have digestive proteases located in the midgut that catalyse the release of peptides and amino acids from the ingested proteins (Jongsma and Bolter, 1997). Lepidopterous and Dipterous insects, such as *C. capitata*, employ serine proteases to digest proteins. These insects have a midgut with an optimum pH for this enzymatic activity (pH 8–11.5). A plant defence mechanism involves the synthesis of protease inhibitors. These inhibitors are proteins that can be constitutively found in many plant organs or can be induced as a response to the attack by herbivorous organisms, acting at the intestinal level of the insect to inhibit the digestion of plant proteins. Although Kalata B1 and B2 do not have any effect on trypsin, chymotrypsin or α amylase of *Helicoverpa*, the cyclotides TI-I and TI-II are the first ones of the trypsin inhibitors subgroup that have been identified (Hernandez *et al.*, 2000) and display homology with a

wide range of plant open-chain trypsin inhibitors (Felizmenio-Quimio, 2001).

As stated before, the known cyclotides are aligned according to the six conserved Cys residues. The loops found in the backbone of these cyclic peptides correspond to the regions located between these residues, and overlapped in these loops are located the amino acidic chains responsible for the biological activities of cyclotides. According to these data, some of the cyclotides present in the 50% EtOH extract of *H. parviflorus* might have, according to its structure, a trypsin inhibitory activity.

Whichever the mechanism of action may be, these findings clearly demonstrate that the outstanding stability of the cystine knot structure makes it an excellent framework on which a wide range of biological activities can be added, generating potential applications as insecticides in agriculture. Furthermore, the ursolic acid and the β-sitosterol that have been isolated from many plant species presented different degrees of anti-alimentary activity on lepidopterous insects. These compounds, present in the CH_2Cl_2 extract of *H. parviflorus*, could be in part or totally responsible for the insecticidal activity of these extracts.

13.6 Potential Uses of Cyclotides

As was observed, the cyclotides have a wide range of biological activities and a high stability owing to their chemical structure. The applicability of linear peptides as drugs is limited because of their susceptibility to proteolytic cleavage and their low bioavailability. The stability of linear peptides can be improved by linking their N and C termini as long as they are relatively close. The pharmaceutical industry has applied cyclization to stabilize the conformation of small linear peptides but not polypeptides and proteins (Craik, 2001). The latter drawbacks could be overcome by employing the cyclotides structure as a scaffolding for the development of novel drugs with different activities (Craik *et al.*, 2002; Barry *et al.*, 2004; Clark *et al.*, 2006). The cyclotides would also have interesting applications in agronomy (Alexander, 2001) because they are potent insecticides and would also serve as models for the development of compounds with potential applications in agriculture (Jennings *et al.*, 2001, 2005).

Cyclic peptides are naturally occuring miniproteins in plants, and their introduction in commercial crops present less technical difficulties than the transference of bacterial genes, e.g. to cotton. According to Göransson and Craik (2003), the gene transference from one plant displaying insecticidal activity to another is a methodology worth exploiting. This technology could be applied to a wide range of crops including cotton, maize, soybean and, potentially, wheat and rice cultures (Johensen and Ho, 2004).

The global expenditure in the chemical control of insect plagues is estimated to be more than 3 billion US dollars per year. By generating these products, a substantial part of the traditional insecticide market would be redirected towards the biotechnological industry, as has occured with the first generation of transgenic plants that currently represent a market of 350 million US dollars.

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14 Natural Polymers and their Biological Activities

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14.1 Basic Polysaccharides

The basic polysaccharides are composed of units of amino sugar usually N-acetylated. These polysaccarides and their derivatives have applications in industry, medicine and biochemistry (Muzzarelli, 1985; Yalpani, 1985). Chemical modification of the amino group of basic polysaccharides allows the introduction of new functional groups that can confer novel properties such as antibacterial, antigenic and anticoagulant activities (Lillo and Matsuhiro, 1997).

14.1.1 Chitosan, chitooligosaccharides and derivatives

Chitosan presents biological activity owing to its biocompatibility. This polysaccharide is a cationic biopolymer obtained by the N-deacetylation of chitin. These polysaccharides are widely distributed in the shells of crustacea (crab, shrimp, etc.), in the cuticles of insects, in the shells and skeletons of molluscs (krill, squid, etc.) and in the cell walls of fungi (mushroom, baker's yeast, etc.). The amino and two hydroxyl groups found in chitosan are targets of chemical modification (Fig. 14.1) (Hirano *et al.*, 1987).

The antimicrobial activity of chitosan against a variety of bacteria and fungi is greater than that of chitin. Its biological activity is based on the presence of the free amino group at carbon 2 in the D-glucosamine units that, in acidic conditions, are charged positively. These groups interact with negative macromolecule residues that are located at the cellular surface of the bacterium, inhibiting its growth.

Chitosan of low molecular weight and oligosaccharides of this biopolymer prepared by acid and enzymatic depolymerization have strong antibacterial and antifungal activities (Kurita, 2001; Kumar *et al.*, 2004). The chitooligosaccharides are prepared through enzymatic hydrolysis using chitonases. The oligosaccharides of a molecular weight in the rank of 10,000 Da present a greater antibacterial activity. The molecular size of the chitooligosaccharides is of great importance in the inhibition of microbial growth (Jeon *et al.*, 2001).

A series of chitooligosaccharide derivatives obtained by chemical modfication had a high antimicrobial activity on *Streptococcus mutans*, the main etiological agent of dental caries in humans. Oligosaccharides of chitosan (COS) were reacted with glycidyl

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trimethylammonium chloride (GTMAC), generating chitooligosaccharides with a quaternary ammonium function (COS-GTMAC) (Fig. 14.2). The derivatives presented a greater antimicrobial activity than the non-modificated oligosaccharides because of the presence of the quaternary group (Kim *et al.*, 2003).

14.2 Biological Activity

Zheng and Zhu (2003) studied the antimicrobial activity of chitosan oligomers with molecular weights smaller than 305 kDa against *Escherichia coli* (a Gram-negative bacterium) and *Staphylococcus aureus* (a Gram-positive bacterium). An increase in concentration and molecular weight of chitosan oligomers increases the antimicrobial effect on *S. aureus*. Chitosan oligomers with a greater molecular weight form a film in the surface of the microbial cell, preventing the adsorption of nutrients. In the case of *E. coli*, a disminution in the molecular weight of the polysaccharide also increases the antimicrobial activity. The chitosans of smaller molecular weight easily enter the external cell membrane, affecting cellular metabolism.

The preparation by enzymatic depolymerization of low molecular weight chitosans in the rank of 4.1–5.6 kDa using papain from *Carica papaya latex* has been reported. The antimicrobial activity of the oligosaccharides obtained was evaluated against *Bacillus cereus* and *E. coli*, and presented an antimicrobial effect greater

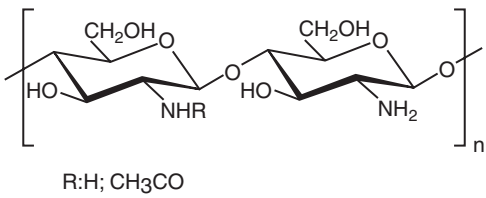

Fig. 14.1. Chitosan structure.

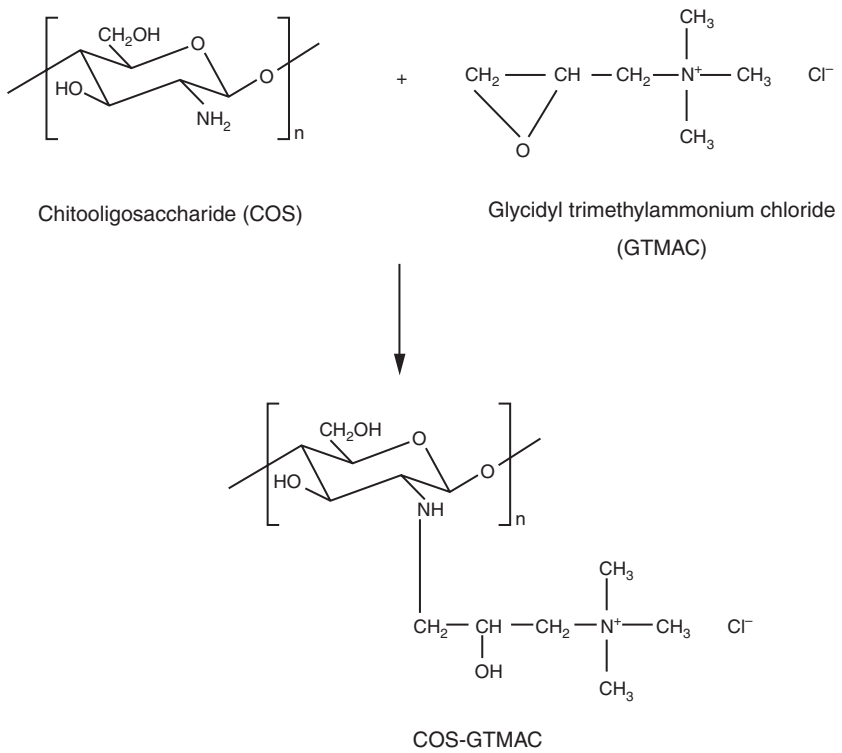

Fig. 14.2. Reaction of chitosan (COS) with glycidyl trimethylammonium chloride (GTMAC).

than that of the native polysaccharide. The antimicrobial activity of the oligosaccharides is superior owing to the increase in the free amino groups by the enzymatic depolymerization (Kumar and Varadaraj, 2004).

Lim and Hudson (2004) synthesized a novel fibre-reactive chitosan derivative in two steps from a chitosan of low molecular weight and low degree of acetylation. First, a water-soluble chitosan derivative, N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC), was prepared by introducing ammonium salt groups on the amino groups of the chitosan. This derivative was modified by the introduction of functional (acrylamidomethyl) groups, which can form covalent bonds with cellulose under alkaline conditions, on the primary alcohol groups (C-6) of the chitosan backbone. The fibre-reactive chitosan derivative, *O*-acrylamidomethyl-HTCC (NMA-HTCC), caused a complete inhibition in proliferation of *S. aureus* and *E. coli* (Fig.14.3). The derivative can be covalently bonded to textile fibres for its use as an antimicrobial agent in the textile industry.

The synthesis of the guanidinylated chitosan derivatives with different molecular weights by the guanidinylation reaction of chitosan with aminoiminomethanesulfonic acid (AIMSOA) has also been reported. The antibacterial activity of guanidinium derivatives was evaluated against *S. aureus, Bacillus subtilis, E. coli and Pseudomonas aeruginosa.* Compared with chitosan, the chitosan derivatives presented a higher antibacterial activity. The antibacterial activity of guanidinylated chitosan enhanced with pH decrease (Hu *et al.*, 2007).

Qin and collaborators (2006) prepared different samples of chitosan with different molecular weights by depolymerization with hemicellulase and water-soluble half N-acetylated chitosan samples by N-acetylation

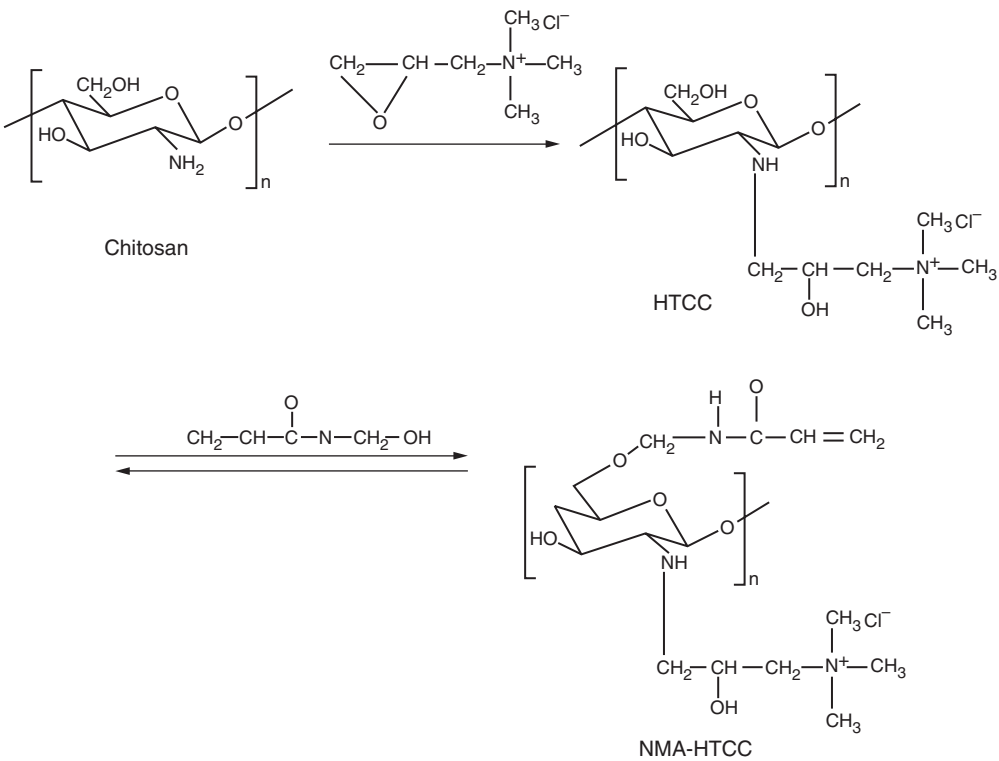

Fig. 14.3. Synthesis of N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) and O-acrylamidomethyl (NMA)-HTCC.

with acetic anhydride. The action of chitosan with molecular weights from 1.4×10^3 to 4.0×10^5 Da against *S. aureus, E. coli* and *Candida albicans* was determined. The water-soluble half N-acetylated chitosans and chitooligomers do not have significant antimicrobial activity. Moreover, water-insoluble chitosan and chitooligomers promote the growth of *C. albicans*. In contrast, water-insoluble chitosans with a molecular weight of around 5.0×10^4 Da present a better antimicrobial action in these tested samples. The antimicrobial mechanism of water-insoluble chitosan was hypothesized to form an impervious layer around the cell.

The antimicrobial activity of chitooligosaccharides with different degrees of deacetylation and polymerization was evaluated on several fungal and bacterial species. The antimicrobial activity of chitooligosaccharides increased with an increase in deacetylation but decreased with an increase in polymerization. The chitooligosaccharides showed a major antimicrobial activity against bacteria rather than fungi. However, the antimicrobial activity of chitooligosaccharides was significantly higher than that of chitosan because the low degree of polymerization of the chitooligosaccharides allows them to penetrate the cell membrane of the microorganisms, interact with DNA in the cytoplasm and lead to a mistake in DNA replication, resulting in the suppression of microbial growth (Wang *et al.*, 2007).

Recently, Lillo *et al.* (2008) studied the antibacterial activity of chitooligosaccharides obtained by partial acid hydrolysis of chitosan. The fraction corresponding to the molecular weight of 10,000 Da was modified by reductive alkylation of the amine group of chitooligosaccharide with D-(+) glucosamine hydrochloride in the presence of sodium cyanoborohydride and afforded the aminoglicosylated derivative shown in Fig. 14.4. This derivative has a prominent antibacterial activity against *S. aureus*.

14.3 Fungal Polysaccharides

The microbial exopolysaccharides (extracellular polysaccharides; EPS) are a class of high-value biopolymers with a variety of industrial applications. Various types of EPS have been used in medicine, foods, cosmetics and other industries. They have potent biological and pharmacological activities, including immune-stimulating, anti-tumour and hypoglycaemic activities. In particular, many kinds of EPS have been produced from submerged cultures of mushrooms or entomopathogenic fungi (Kim *et al.*, 2003).

The exopolysaccharide of *Paecilomyces tenuipes* C240 is highly valued because of its various biological and pharmacological activities including its immuno-stimulating and antitumor activities (Xu *et al.*, 2003). Although many studies have examined the effect of culture conditions on the production of microbial polysaccharides, little is known about the influence on the product quality, particularly molecular characteristics. Several investigators have pointed out that culture medium and environmental conditions affect the production and the physico-chemical characteristics of exopolysaccharides (Xu and Yun, 2004; Xu *et al.*, 2006).

Takagi and Kadowaki (1985) optimized the submerged culture conditions to produce the exopolysaccharide 1→4-2-amino-2-deoxy-α-D-galactan (Fig. 14.5), also known as poly-α-D-galactosamine, from the fungi *Paecilomyces* sp. I-1. Poly-α-D-galactosamine may constitute an important starting material for fine chemicals and biologically active derivatives. It is known that it exhibits anti-tumoural effects and bacterial activity. It shows similar physicochemical properties to chitosan, but the easy production of this polysaccharide, and its stability against enzymes or microorganisms capable of hydrolysing glucosamine residues, are advantages over chitosan.

Lillo and Matsuhiro (2003) studied the growth kinetics of *Paecilomyces* sp. and the production of EPS. The major production of EPS is obtained in four-day cultures. On the other hand, the concentration of the EPS is inversely proportional to the increase in the biomass of the fungus. This decrease in the production of EPS probably could be due to the exhaustion of the carbon source in the

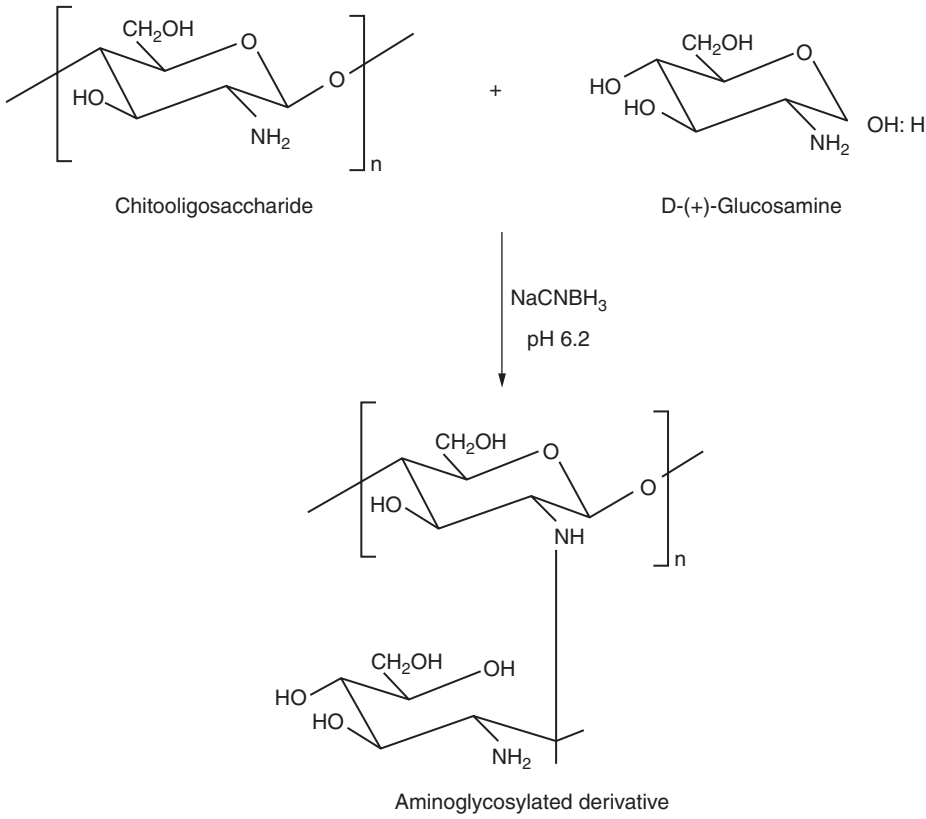

Fig. 14.4. Reductive alkylation of chitooligosaccharide to obtain the aminoglycosylated derivative.

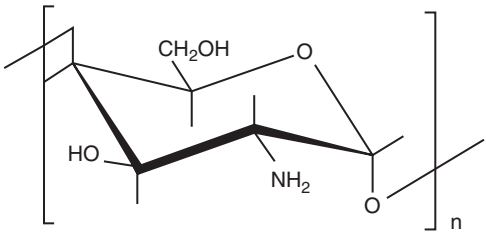

Fig. 14.5. 1→4-2-amino-2-deoxy-α-D-galactan (poly-α-D-galactosamine).

culture medium. The growth kinetics of *Paecilomyces* sp. and the production of EPS are shown in Fig. 14.6.

On the other hand, the exopolysaccharide of *Paecilomyces* sp. was analysed by gel permeation chromatography, which revealed the existence of a homogeneous EPS. The molecular weight was estimated to be about 700 kDa. The constituent monosaccharides were determined by means of acid hydrolysis of the polymer and later chromatographic analysis and spectroscopy (Lillo *et al.*, 2007).

Huber and collaborators (1984) determined the composition of the major acid EPS of 25 strains of *Rhizobium japonicum*. Eight strains synthesized an acidic EPS containing rhamnose and 4-*O*-methylglucuronic acid and were closely related according to DNA homology. The same strains also expressed high levels of *ex planta* nitrogenase activity. Of the 25 strains, 16 produced an acidic EPS containing glucose, mannose, galacturonic acid and galactose, and were also related by DNA homology. These strains developed little or no nitrogenase activity under the experimental conditions employed.

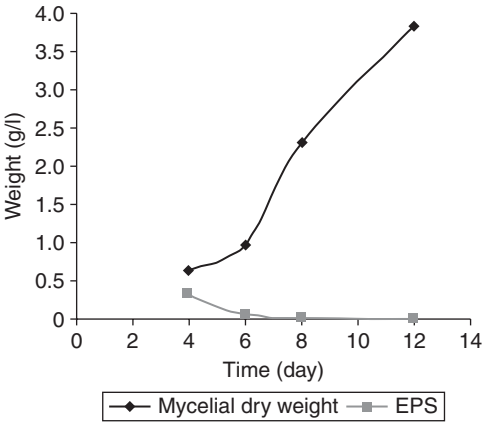

Fig. 14.6. Growth kinetics of *Paecilomyces* sp. and the production of exopolysaccharides (EPSs).

The EPS produced by submerged culture of *Phellinus gilvus* has notable biological activities such as anti-tumour activity, free-radical scavenging activity and proliferation activity of the human fibroblast cells. Furthermore, *P. gilvus* produced a higher concentration of EPS and mycelial biomass in comparison with other *Phellinus* species (Hwang *et al.*, 2003).

Acknowledgements

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15 Insect Growth Regulatory, Moulting Disruption and Insecticidal Activity of *Calceolaria talcana* (Calceolariaceae: Scrophulariaceae) and *Condalia microphylla* Cav. (Rhamnaceae)

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15.1 Introduction

Plants produce a great variety of secondary metabolites that do not have apparent function in physiological or biochemical processes; these compounds (or allelochemicals) are important in mediating interactions between plants and their biotic environment (Berenbaum, 1989, 1991, 1995, 2002; Kessler and Baldwin, 2002). Some of them can be used as leader molecules for the development of protective agents against insects and fungi (Kubo *et al*., 1981, 1993, 2000, 2003a,b; Crombie, 1999), and enzyme inhibitors (Kubo, 1997; Keane and Ryan, 1999; Ortego *et al*., 1999; Kubo *et al*., 2000, 2003a,b; Céspedes *et al*., 2001a,b). The increasing interest in the possible application of secondary metabolites for pest management has directed the investigation towards the search for new sources of biologically active natural products, with new modes, sites and mechanisms of action (Gonzalez-Coloma *et al*., 1997; Conner *et al*., 2000; Eisner *et al*., 2000; Meinwald, 2001). These characteristics may enhance their value as commercial pesticides (Gonzalez *et al*., 1992, 2000; Valladares *et al*., 1997; Gonzalez and Estevez-Braun, 1998; Isman *et al*., 2006; Akhtar *et al*., 2008).

In this chapter we review the results of our research group on the bioactivity of extracts and secondary metabolites from *Calceolaria talcana* and *Condalia microphylla*.

15.2 *Calceolaria talcana*

Plants from the genus *Calceolaria* (Scrophulariceae) are distributed in temperate and tropical regions of New Zealand and Central and South America. (Engler, 1964; Di Fabio *et al*., 1995; Garbarino *et al*., 2004). Several species of *Calceolaria* are used as

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ornamental plants and in traditional medicine (Falcao *et al*., 2006). The aerial parts of these plants are used in Chile owing to their analgaesic, digestive and diuretic properties (Sacchetti *et al*., 1999), and as an antimicrobial in stomach treatment (Sacchetti *et al*., 1999; Garbarino *et al*., 2004). Some species of this genus have substances with potential use as: insecticides (Khambay; Jewess, 2000), against tuberculosis (Woldemichael *et al*., 2003) and as growth inhibitors of TA3 tumour cells and methotrexate resistant TA3 cells (Morello *et al*., 1995). Flavonoids, glucophenylpropanoids and diterpenes were identified in *Calceolaria* (Wollenweber *et al*., 1989; Di Fabio *et al*., 1995; Nicoletti *et al*., 1998; Garbarino *et al*., 2000). There are around 86 species growing in Chile (Marticorena *et al*., 1985) and only 15% of them have been phytochemically characterized.

Calceolaria talcana Grau & C. Ehrhart (Calceolariaceae: Scrophulariceae), an endemic from Chile, belongs to the *Calceolaria integrifolia sensu lato* complex. The latter comprises nine species: *Calceolaria andina, Calceolaria angustifolia, Calceolaria auriculata, Calceolaria georgiana, Calceolaria integrifolia s.str, Calceolaria rubiginosa, Calceolaria talcana, Calceolaria verbascifolia* and *Calceolaria viscosissima*. Each one of these species has its own characteristic distribution pattern, which correlates with ecological and weather factors (Ehrhart, 2005). Growing between the VII to VIII region together with other species of *Calceolaria, C. talcana* (Table 15.1), commonly known as 'zapatito de doncella' or 'capachito de hoja larga', is a strong erect shrub, 150 cm tall or smaller with fragile ascending branches, internodes of 2–8 cm, and the inflorescence and distal parts of stems are glutinous or velutinous with erect hairs (Fig. 15.1a) (Ehrhart, 2005).

Table 15.1. Taxonomy of *Calceolaria talcana*.

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Scrophulariales
Family	Scrophulariaceae
Genus	Calceolaria
Species	*Calceolaria talcana*

The phytochemical study of the extracts of the *C. talcana* species has led us to find a relationship between them and the extracts of *Penstemon gentianoides* and *Penstemon campanulatus*; they are also Scrophulariaceae and the phytochemicals found can contribute to chemotaxonomy studies of this species. *P. campanulatus* is an evergreen shrub that grows throughout high mountains in Guatemala, Mexico and the southern states of the USA. Its leaves and roots have been used therapeutically for anti-inflammation, and we have some reports about this activity (Dominguez *et al*., 2010) and antioxidant activity, among others (Dominguez *et al*., 2005). Thus, in a parallel manner, identical phenylethanoids, iridoids and flavonoids to those in *Calceolaria* have been identified from its aerial parts (Dominguez *et al*., 2007; Muñoz *et al*., 2013).

15.3 *Condalia microphylla*

Condalia microphylla Cav. (Rhamnaceae) is known in Chile and Argentina as 'Piquilin' (Fig. 15.1b). This densely branched shrub occurs on arid lands in central Argentina ('Montane grasslands and shrublands') and in the foothills of north–central Chile ('Mediterranean forests woodlands and shrublands'). It grows to approximately 2 m tall. Although this shrub has not been studied in our country, the presence of hydrocarbons and fatty acids has been reported in the *Condalia montana* species complex (Zygadlo *et al*., 1991, 1992).

Condalia microphylla causes 'el mal del piquilin' in cattle, a neurotoxic or hepatotoxic disease (Bedotti *et al*., 2006). The toxicological activity of *Condalia* species has also been reported by Delgado *et al.* (2011). Additionally, the use of aerial parts (bark, leaves and stems) induces the accumulation of long-chain *n*-alkanes, producing ataxia and 'paraffin-liver' in cattle

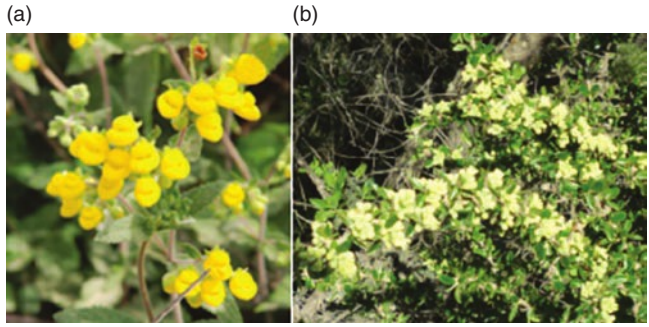

Fig. 15.1. Flowers and leaves of (a) *Calceolaria talcana* and (b) *Condalia microphylla.*

(Halse *et al.*, 1993; Delgado *et al.*, 2011). Several *Condalia* species (i.e. *Condalia buxifolia*) contain cyclopeptide alkaloids showing several biological activities (Morel *et al.*, 2002). The bark and root of *C. microphylla* have tannins used as dyes for the coloration of handcrafts (Gimenez *et al.*, 2008).

Together, the information provided above reveals that *C. microphylla* produces several bioactive metabolites. Based on this and the high resistance of the wood and leaves to insect and pathogen attack, further studies have been carried out to determine insecticidal and insect growth regulatory effects of the methanol, *n*-hexane, ethyl acetate and aqueous extracts of aerial parts (Céspedes *et al.*, 2013).

15.4 Materials and Methods

15.4.1 Plant materials

Calceolaria talcana Grau & C. Ehrhart was collected 4.7 km NW of Confluencia, on the north shore of the Itata river (36° 37' 21" S, 72° 28' 16" W elevation 170 ft) Ñuble province, VIII region, Chile, in November 2010. Voucher specimens have been deposited in the Herbarium of the Basic Science Department, University of Bío-Bío (Voucher DS-2010/05-16243/44) and in the Herbarium of the University of Illinois, at Urbana-Champaign, USA, (ILL, Voucher DS-16243/44). The samples were identified by Prof. David S. Seigler, PhD (Full Professor Plant Biology Department and Curator Herbarium of University of Illinois at Urbana-Champaign). Samples of aerial parts were dried, milled and then macerated, and concentrated under vacuum.

Samples of aerial parts (leaves, heartwood and bark) of *Condalia microphylla* Cav. were collected during spring 2010 (November) on the slopes of the Andes Mountains near Los Andes City in Chile (collection 1) and at the Ecological Reserve on Campus of Catholic University of Cordoba, Cordoba, Argentina (collection 2). Voucher specimens were deposited in the Herbarium of Basic Science Department, University of Bio-Bio, Chillán, Chile (Voucher CLC/0035) and Herbarium 'Dr. Marcelino Sagayo' (Faculty of Agricultural Sciences, Catholic University of Cordoba, Argentina). Specimens were identified by Prof. Gustavo Ruiz, PhD, Director of the Herbarium. The samples of *C. microphylla* were dried and milled, then were macerated with methanol and concentrated under vacuum rotatory evaporation until a viscous consistency.

15.4.2 Extracts and fractions

The plants were dried at room temperature, crushed and then immersed overnight in methanol. The next day the methanol was filtered, and the extraction process was repeated three times. The filtrate was concentrated at reduced pressure in a rotary evaporator at 40°C and 200 mb.

The MeOH extracts were re-dissolved with MeOH/H_2O (6:4) and then these solutions were deposited in a decantation funnel. Twenty extractions were made, each one with 150 ml of hexane. The hexane phase was gathered and concentrated at reduced pressure. The process was repeated with ethyl acetate, subsequently leaving a residue MeOH/water (Fig. 15.2).

15.4.3 Determination of the total phenolic content

The content of total phenolic compounds was measured using a method described previously by Céspedes *et al.* (2010). An aliquot (1 ml) of the proper diluted extract was added to 1 ml of half diluted Folin-Ciocalteu reagent (Sigma-Aldrich, Santiago, Chile). To this was added 2 ml of 20% Na_2CO_3 10 min later. After 5 min the absorbance was measured at 730 nm with a UV2310 Techcomp multichannel spectrophotometer. Additionally, 10 µl of sample or standard (10–100 µM catechin) plus 150 µl of diluted Folin-Ciocalteu reagent (1:4 reagent/water) was placed in each well of a 96-well plate and incubated at room temperature for 3 min. Following the addition of 50 µl of sodium carbonate (2:3 saturated sodium carbonate/water) and a further incubation of 2 h at room temperature, absorbance was read at 725 nm with a BIOTEK Epoch microplate spectrophotometer equipped with Gen5™ microplate data analysis software. Results are expressed as micromoles of Cat E per gram using catechin as the standard (Dominguez *et al.*, 2005). All tests were conducted in triplicate.

Yield of verbascoside and total phenolic compounds were expressed as the amount of each component extracted per gram of dried extract of *Calceolaria*.

15.4.4. Antifeedant test against *Spodoptera frugiperda*

The antifeedant activity of the extracts was evaluated against *Spodoptera frugiperda* larvae according to the methodology used by Valladares *et al.* (1997). Briefly, one larva per Petri dish was deposited with two lettuce circles of 1 cm^2, one sprayed with 10 µl of extract dissolved in ethanol at a 100 ppm concentration, and the other sprayed with the same quantity of ethanol. Under the lettuce, a round piece of paper towel moistened with distilled water was placed to reduce the dehydration of the plant material. Ten repetitions were made for each extract and ten repetitions for the *n*-hexane extract in each one of the concentrations used. The measurements were made for 24 h, establishing the percentage of consumed area (estimated visually through the use of a grid), and the antifeedant index rate was calculated, AI% = [(1–T/C) × 100], T being the average food consumption treated with extract and C the equivalent in the controls. Once the fractions that had a higher AI% were determined, a gradient was made in the concentrations of these extracts and its effect was measured for 24 h.

15.4.5 Insecticidal bioassay against *S. frugiperda*

The *S. frugiperda* (J.E. Smith) larvae used for this experiment were maintained under

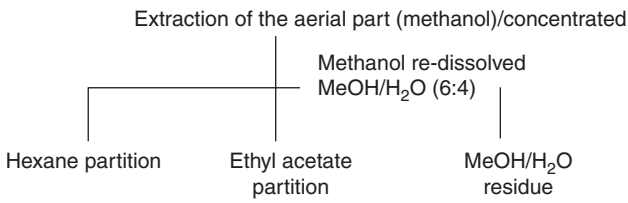

Fig. 15.2. Method of obtaining extracts, partitions and fractions.

conditions described previously (Céspedes *et al.*, 2000). Briefly, the artificial diet (1 kg) used contained 800 ml of sterile water, 10.0 g of agar, 50.0 g of soya meal, 96.0 g of corn meal, 40.0 g of yeast extract, 4.0 g of wheat germ, 2.0 g of sorbic acid, 2.0 g of choline chloride, 4.0 g of ascorbic acid, 2.5 g of p-hydroxybenzoic acid methyl ester, 7.0 ml of Wesson salt mixture, 15.0 ml of Vanderzant vitamin mixture for insects, 2.5 ml of formaldehyde, 0.1 unit of streptomycin, 5.0 g of aureomycin and 20.0 g of milled ear of maize grain, which was prepared by the procedure described earlier (Céspedes *et al.*, 2000). Polystyrene multidishes (24-well) were filled with the liquid diet and allowed to solidify for 20 min at room temperature under sterile conditions. The 3.4 ml wells measured 17 mm in depth and 15 mm in diameter, with a 1.9 cm^2 culture area. All test extracts were dissolved in 95% ethanol and layered on top of each well with the artificial diet at five concentrations, using 1 ml 95% ethanol as a control. For each concentration used and for the controls, a single *S. frugiperda* neonate larva was placed on the diet mixture in each well for 7 days. Thus each treatment included 72 larvae in total (i.e. three plates of 24 wells). After 7 days, surviving larvae were measured and weighed and then transferred to separate vials containing fresh stock diet. Larval weight gains and mortality were recorded after 21 days of incubation because the pupation average is 23 ± 1 day. Some life-cycle data such as time to pupation, mortality of larvae and adult emergence and deformities were registered. All experiments were carried out in a controlled environmental chamber with an 18:6 light:dark (L:D) photoperiod, 19 and 25°C night and day temperature, respectively, and a relative humidity of 80 ± 5%. There were three replicates for each treatment. Controls contained the same numbers of larvae, volume of diet and ethanol as the test solutions (Céspedes *et al.*, 2000, 2005; Torres *et al.*, 2003; Céspedes and Alarcon, 2011).

15.4.6 Not-choice test against *Drosophila melanogaster*

Insecticidal activity against larvae of *Drosophila melanogaster* was assayed as follows (Miyazawa *et al.*, 2000): five concentrations (10.0, 20.0, 50.0 and 100.0 ppm of sample) were used determining LD_{50} values. Test compounds were dissolved in 50 µl of EtOH and mixed in 1 ml of artificial diet (60 g of brewer's yeast, 80 g of glucose, 12 g of agar, 8 ml of propionic acid and 1000 ml of water). A control diet was treated with 50 µl of EtOH only.

The diet and 10 eggs were placed in a Petri dish. Ten Petri dishes were prepared. Then, they were placed at 25°C and relative humidity >90% for 8 days. One day after transplantation, the larvae hatched and were fed with the different test extracts (water, ethyl acetate, hexane and methanol) in the different concentrations (10.0, 20.0, 50.0 and 100.0 ppm of sample) mixed with the artificial diet. At 25°C, larvae generally change to pupae after 7 days. In each instance the developmental stage was observed, and the numbers of pupae were recorded and compared with those of a control.

15.4.7 Bioassays with yellow meal worm (*Tenebrio molitor*)

Larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were fed with wheat bran in plastic boxes at 24.0 ± 1°C, with a 16:8 L:D photoperiod, these larvae maintained in a chamber under these environmental conditions were used in the test. Bioassays were performed with last instar larvae of *T. molitor* based on live weight (103–160 mg). For each compound, test solutions Me_2CO/MeOH (9.5:0.5 v/v) were topically applied to ventral abdominal segments with a microsyringe 2 µl/larva; equivalent to 0.2 µg/larva of the assayed compounds for each one of concentrations assayed. Controls were treated with the solvent alone. For each individual compound there were three replicates of 20 larvae each, and the assay had three replicates. After treatment the insects were placed in Petri dishes

(5 cm diameter) with 3 g of sterilized wheat bran, a plug of moistened cotton to preserve humidity and held at 24.0 ± 1°C with 16:8 L:D photoperiod. The number of larvae that successfully pupated, as well as the duration of the pupal stage (in days), were recorded every 24 h for 30 days (end point of the experiment) (Céspedes *et al.*, 2005).

15.4.8 Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used in assays to measure the antioxidant activity of the extracts from *C. talcana* (Mensor *et al.*, 2001). A stock solution of DPPH was prepared (0.03 g/l of DPPH in methanol) from which 5 ml were mixed with 100 µl of each one of the extracts of *Calceolaria* (ethyl acetate, methanol, *n*-hexane and aqueous) in concentrations from 50 ppm to 300 ppm. The samples of *C. microphylla* did not show significant antioxidant activity.

Each sample was stored in a dark room for 30 min at room temperature, and then the absorbance was measured at 517 nm. The control corresponded to the measure of the solvent in which is dissolved each extract at 517 nm. As a negative control the same mixture was used as described previously but without including the extracts and, as a positive control, quercetin, α-tocopherol and an ethyl acetate extract from tecu-berry fruit (*Aristotelia chilensis*; Céspedes, *et al.*, 2010) were used. Both samples' absorbance was measured at 517 nm.

15.4.9 Statistical analyses

Data shown are average results obtained by means of five replicates and are presented as average ± standard errors of the mean (SEM). Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by generalized linear model (GLM) procedures. Results are given in the text at $p < 0.05$. Differences between means were established with a Student–Newman–Keuls (SNK) test. The LD_{50} values were calculated by PROBIT analysis based on percentage of mortality obtained at each concentration of the samples. Complete statistical analysis was performed by means of the MicroCal Origin 6.0 statistical and graphs PC program.

15.5 Results and Discussion

15.5.1 *Calceolaria talcana*

C. talcana and *C. microphylla* were incorporated in our screening programme designed to discover interesting biological activities of plants from template regions. Both species showed relevant insecticidal, insect-growth-regulatory (IGR) and antioxidant activities. On the basis of this information, and the high resistance to insect and pathogen attack exhibited by these plants, we investigated the IGR activity of their *n*-hexane, ethyl acetate and MeOH/aqueous extracts. Extracts of *C. talcana* showed very satisfactory insecticidal and IGR activities. Some bioassays were performed at concentrations lower than 10 ppm. In addition to these extracts, gedunin, and the methanolic extracts from *Cedrela salvadorensis* (Me-Ced), *Myrtillocactus geometrizans* (Me-Myrt) and *Yucca periculosa* (Me-Yuc), were used as positive controls (Céspedes *et al.,* 2000, 2005; Torres *et al.*, 2003).

A milled sample from *C. talcana* was macerated with MeOH and further partitioned with *n*-hexane, ethyl acetate and water, respectively. These extracts were used in a preliminary bioassay trial. Subsequently, in order to obtain more satisfactory data for the insecticidal activity, we used concentrations as low as possible so that there was no effect owing to the toxicity of the samples to many targets that may not be specific to the insect but general or broad spectrum for plants, insects and humans.

15.5.2 Verbascoside and total phenolics

The highest concentration of verbascoside was found in the ethyl acetate extract

(72.3 mg/g extract), whereas the concentration in the residue of methanol/water was 13.1 mg/g extract. The *n*-hexane did not show the presence of this compound.

15.5.3 Antifeedant activity

Some of the extracts of *C. talcana* showed a high feeding dissuasive activity over the *S. frugiperda* larvae in the choice assays (Table 15.2). The consumption of leaves treated with methanol, ethyl acetate and MeOH-Yuc extracts were significantly lower than for *n*-hexane and control, showing high levels of feeding inhibition according to the established values (Hassanali and Lwande, 1989). Significant feeding inhibition occurs when the antifeedant index (AI) is greater than 75% and moderate when it is between 50 and 75%. At a concentration of 50 µg/ml, verbascoside, gedunin, ethyl acetate and methanol extracts showed a significant AI reduction in leaf consumption and this was 96.7, 89.9, 89.7 and 88.3%, respectively, while at 25 µg/ml the AI was 88.2, 85.6, 75.5 and 71.3% for verbascoside, gedunin, ethyl acetate and methanol extracts, respectively (Table 15.2).

The most active extract against *D. melanogaster* was ethyl acetate, which showed high deterrence power (Table 15.3), even at concentrations of 25 µg/ml (75.1%), whereas the percentage was reduced to moderate effects for the methanol extract. Verbascoside and gedunin show the highest effect with 89% and 81% at 10 µg/ml, respectively. At concentrations greater than 10 µg/ml both compounds produced 100% AI (Table 15.3).

15.5.4 Insecticidal activity against *D. melanogaster*

The results obtained in the tests of not-choice for each one of the extracts of *C. talcana*

Table 15.2. Results obtained in antifeedant tests, using different extracts and compounds at different concentrations of *C. talcana* on *S. frugiperda* larvae.

Concentration (µg/ml)	AI(%)* of extracts					AI(%) compounds	
	Aqueous	Ethyl acetate	Hexane	Methanol	Me-Yuc	Verbascoside	Gedunin
100	–78.6	100a	69.1e	100a	100a	100a	100a
50	–41.1	92.3b	55.6f	88.34c	77.9d	100a	89.9c
25	–30.4	89.7c	33.2g	71.30d	n.d.	96.7b	85.6c
10	–9.3	75.5d	20.2g	67.45e	n.d.	88.2c	74.5d
5	0	69.9e	19.9g	45.33f	n.d.	78.9c	68.8e

*AI(%) = Antifeedant index = [(1–T/C) × 100]. n.d., not determined. The values followed by the same letter are not significantly different. The significance level is p <95%.

Table 15.3. Results obtained in antifeedant tests, using different concentrations of extracts from *C. talcana* on *D. melanogaster* larvae.

Concentration (µg/ml)	AI(%)* extracts					AI(%) compounds	
	Aqueous	Hexane	Methanol	Ethyl acetate	MeOH-Yuc	Verbascoside	Gedunin
100	–78.9	92.31b	100a	100a	100a	100a	100a
50	–45.0	87.50c	93.67b	100a	85c	100a	100a
25	–31.5	49.00f	55.34f	75.1d	n.d.	100a	98a
10	–10.8	–30.30	10.00g	56.5f	n.d.	89c	81c

*AI(%) = Antifeedant index = [(1–T/C) × 100]. n.d., not determined. The values followed by the same letter are not significantly different. The significance level is p <95%.

shows that three of four extracts possesses a lethal effect on the *D. melanogaster* larvae (Table 15.4). Against *D. melanogaster,* verbascoside, gedunin, the extracts of ethyl acetate and methanol at 20 µg/ml showed 92.0, 87.1, 67.0 and 65.0% of mortality effect, respectively, and at concentrations of 50 and 100 µg/ml the percentage of mortality of the larvae after 72 hours was 100% for verbascoside, gedunin, ethyl acetate and methanol extracts.

15.5.5 Insecticidal activity against larvae of *S. frugiperda*

The effects of methanol extracts of *C. talcana* on growth and development of larvae of first instar of *S. frugiperda* was evaluated initially at a concentration of 100 µg/ml. The results for *S. frugiperda* larvae are outlined in Table 15.5. Interestingly, at 21 days the number of larvae and pupae decreased drastically in all treatments. For example, at a concentration greater than 35.0 µg/ml for *n*-hexane, 20.0 µg/ml for ethyl acetate and to concentrations greater than 10.0 µg/ml for methanol extracts the pupation was very low (Table 15.5). When pupation did occur serious abnormalities were observed, and this phenomenon was observed in similar form to *D. melanogaster*, ecdysis and sclerotization were incomplete. Ultimately, all pupae under ethyl acetate and methanol treatments died at concentrations above 35.0 µg/ml (Table 15.5).

In experiments of not-choice carried out against larvae of first instar of *S. frugiperda* and neonate larvae of *D. melanogaster* during the first 6 days, the effects of the ethyl acetate and methanol extracts were 100% lethal at concentrations greater than 35.0 and 20.0 µg/ml, respectively, whereas verbascoside and gedunin were 100% lethal at concentrations greater than 10.0 and 50.0 µg/ml, respectively. The 95% lethal doses (LD_{95}) of these extracts against *S. frugiperda* are: methanol (20.0 µg/ml), hexane (95.0 µg/ml) and ethyl acetate (20.0 µg/ml). Thus, methanol, ethyl acetate extracts and verbascoside all exhibited 100% larval mortality and gave the highest insecticidal activity. It is important to point out that ethyl acetate, possessing an LD_{95} of 20.0 µg/ml, was more active as an insecticide than gedunin or any of the three extracts (Me-Ced, Me-Myrt and Me-Yuc) used as positive controls.

15.5.6 Insect growth inhibitory activity for *S. frugiperda* larvae

At intermediate concentrations of extracts (10.0 to 35.0 µg/ml) specific inhibition of each larval growth stage, e.g. growth and weight gained (up to 75% of length), was observed (Table 15.5). Moreover, ethyl acetate extract produced the strongest inhibition (11.5 and 11.1%, at 10.0 and 20.0 µg/ml, respectively) of growth and weight increase

Table 15.4. Mortality percentage of *D. melanogaster* larvae, after 72 h of the application of the extracts at different concentrations in larvae's diet.

Extract	Concentration (µg/ml)	Mortality (%)	LC_{50}
	control	0.0	
Aqueous	10	0.0	
	20	0.0	
	50	0.0	
	100	0.0	
Ethyl acetate	10	13.3 ± 0.6a	17.25
	20	67.0 ± 0.6b	
	50	100 ± 1.0c	
	100	100 ± 1.0c	
Hexane	10	7.0 ± 0.6a	43.2
	20	13.3 ± 0.6a	
	50	63.3 ± 0.6b	
	100	100 ± 1.0c	
Methanol	10	30.0 ± 1.5b	15.5
	20	65.0 ± 3.4b	
	50	100 ± 4.47c	
	100	100 ± 4.47c	
MeOH-Yuc	20	55.5 ± 1.9b	17.04
	50	90.5 ± 2.9c	
	100	100 ± 3.7c	
Verbascoside	20	92.0 ± 2.7c	8.08
	50	100 ± 1.7c	
	100	100 ± 1.1c	
Gedunin	20	87.1 ± 3.9c	11.93
	50	100 ± 2.0c	
	100	100 ± 1.1c	

Each value corresponds to the average of the three different experiments ± SE. The values followed by the same letter are not significantly different. The significance level is p <95%. The LC_{50} are expressed as µg/ml.

Table 15.5. Activities of extracts from *Calceolaria talcana* and verbascoside compared with gedunin and pattern extracts on growth bioassay[a], pupation[b] and emergence[b] parameters of *Spodoptera frugiperda* (after 21 days of incubation).

Treatment	Doses (ppm)	Mean weight gained (mg)[c]	%[d]	Pupation[c] SP (%)[e]	Emergence (%)[f]	Mortality (%)[d]
Control[g]	0.0	520.5 ± 16.6a	100.0	97.2	100.0	4.2
Aqueous	10.0	522.9 ± 23.0a	95.8	92.8	90.0	0.0
	50.0	499.4 ± 12.4a	95.9	91.2	90.0	0.0
Hexane	10.0	478.9 ± 16.8a	92.0	92.5	74.2	33.3
	20.0	390.0 ± 12.6a	76.6	76.7	51.2	41.6
	35.0	240.6 ± 21.1g	46.2	32.1	32.5	63.0
	50.0	95.6 ± 7.8c	18.4	11.2	20.8*	72.2
	100.0	27.7 ± 5.6c	5.3	4.9*	0.0	100.0
Ethyl acetate	10.0	381.5 ± 15.1a	11.5	27.8	20.0*	58.3
	20.0	240.1 ± 12.3g	11.0	14.2	18.0*	95.8
	35.0	195.9 ± 12.2d	36.6	8.8*	0.0	100.0
	50.0	52.7 ± 0.9c	11.5	3.1*	0.0	100.0
Methanol	10.0	191.3 ± 12.1d	37.3	12.5*	3.1*	50.0
	20.0	50.1 ± 1.3c	11.1	9.2*	0.0	95.8
	35.0	40.0 ± 0.3e	8.0	4.9*	0.0	100.0
	50.0	22.7 ± 0.3e	4.9	3.1*	0.0	100.0
Verbascoside	10.0	9.2 ± 0.5f	2.15	3.1*	0.0	81.3
	20.0	7.2 ± 0.7f	1.35	3.1*	0.0	100.0
	35.0	0.0	0.0	0.0	0.0	100.0
	50.0	0.0	0.0	0.0	0.0	100.0
Gedunin	10.0	9.86 ± 0.55f	2.05	54.2b	15.0	11.6
	25.0	6.50 ± 0.19f	1.35	41.7b	13.0	8.4
	50.0	0.0	0.0	0.0	0.0	100.0
Me-Ced	2.0	421.1 ± 22.50a	87.45	54.2b	15.0	71.0
	10.0	289.1 ± 14.90g	60.04	41.7b	13.0	52.3
	25.0	166.6 ± 7.83d	34.60	33.3c	8.0	36.3
	50.0	101.2 ± 4.51d	21.01	25.0c	0.0	21.1
Me-Myrt	100.0	240.6 ± 25.1g	46.2	5.0	0.0	63.0
	200.0	95.6 ± 6.8c	18.4	0.0	0.0	72.2
	300.0	65.5 ± 3.7c	12.6	0.0	0.0	97.2
Me-Yuc	10.0	111.0 ± 5.55b	23.05	35.2	6.12	63.0
	15.0	45.0 ± 2.25e	9.35	18.5	0.0	53.1
	25.0	23.0 ± 1.15e	4.77	6.8	0.0	81.0
	50.0	0.0	0.0	0.0	0.0	100.0

[a]The values for growth bioassay were from weight, values taken at 21 ± 1 days before pupation, the criterion followed was to account larvae that formed pupae; the larvae that did not form pupae were counted as dead larvae. [b]Values taken after pupation. The values for aqueous extract were omitted because they are not significant and this sample did not show any effect at all assayed concentrations. [c]Means followed by the same letter within a column after ± standard error values are not significantly different in a Student-Newman-Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% confidence limits. Mean of three replicates. [d]Percentage with respect to control. [e]SP, Survival Pupation = Number of surviving pupae × 100 / Total larvae for pupation. [f]% = Number of adults emerged × 100 / Total number of pupae. *The asterisks indicate deformities. [g]As a control, a normal diet with solvent only and Tween 20 was added to an additional control solution.

at 21 days. On the other hand, the two extracts (ethyl acetate and methanol), above 35.0 µg/ml, showed a high growth inhibition, and after 21 days these extracts showed 100% of mortality, respectively.

The percentage of larvae that reached pupation decreased drastically with almost all extracts assayed (except the aqueous ones). Thus, hexane (35.0 µg/ml, 32.1%), ethyl acetate (10.0 µg/ml, 27.8%) and methanol

(10 µg/ml, 12.5%) extracts showed significant delay of pupation (Table 15.5). Above 35 µg/ml, no larvae emerged to pupation with ethyl acetate and methanol extracts. Delays in time to pupation (>24 days) for hexane (>35.0 µg/ml), ethyl acetate (>10 µg/ml) and methanol (>10.0 µg/ml) were observed. Furthermore, concentrations of ethyl acetate and methanol between 10.0 and 20 µg/ml significantly reduced pupal weights. Ethyl acetate was the extract that produced the greatest effect on pupal weights above 1.0 ppm (data not shown), whereas Me-Ced, Me-Myrt and Me-Yuc extracts produced the greatest effect on pupal weights at 10.0 ppm, as previously reported (Céspedes *et al.*, 2000, 2006; Torres *et al.*, 2003).

The percentage of emergence of adults from the pupae was also drastically affected by these extracts. The greatest reductions were shown with ethyl acetate (35.0 and 50 µg/ml, 100%), and methanol (20–50 µg/ml, 100%), and in almost all could be observed deformities. Thus, at higher concentrations the most active extracts from *Calceolaria talcana* completely blocked the percentage of adult emergence, because no viable adults emerged from the pupae at this step.

During insect development the shedding of the cuticle, known as moulting, or ecdysis, occurs. Moulting affects the entire body wall and all internal parts that are formed as invaginations of the wall. Collectively, all changes that involve growth, moulting and maturation are known as morphogenesis. The moulting process begins when epidermal cells respond to hormonal changes by increasing their rate of protein synthesis. The first step of moulting is apolysis: the separation of epidermal cells from the inner surface of the old endocuticle and the formation of the subcuticular space. A moulting gel (including enzymes) is secreted into this space. An insect larva that is actively constructing new exoskeleton is said to be in a pharate condition (Marks, 1980). In this study, analysis of the test insect fed with *C. talcana* extracts revealed a developmental disruption in which the insects died (between 10 to 50 ppm) during pharate conditions after initiation of moulting (the apolysis step), without completion of morphogenesis.

During a moult, ecdysteroid levels first rise to stimulate onset of apolysis and cuticle synthesis but then must fall to facilitate the release of eclosion hormone (EH) (Truman *et al.*, 2002) and the ecdysis-triggering hormone (ETH) (Zitnan *et al.*, 1999). These last substances act in concert to trigger insect ecdysis during the final stages of the moult. Methanol and ethyl acetate extracts and the major component of the ethyl acetate extract verbascoside may have the effect of disrupting ecdysteroid metabolism to result in an inhibition of emergence behaviour, or may, alternatively, act directly to inhibit the release of ETH (Hesterlee and Morton, 1996), as was observed.

15.5.7 Growth index and relative growth index for *S. frugiperda*

Larvae that reached pupal stage belonged to the lowest concentration groups, and the pupae that emerged showed many deformities. Thus, in all treatments the average time to reach the mean weight of the adult stage relative to the time needed for control larvae to reach the adult stage was significantly delayed. The growth index (GI or number of surviving larvae/total larvae used) and relative growth index (RGI or GI treated/GI control; Zhang *et al.*, 1993) showed (Table 15.6) that the strongest effects were at 35.0 µg/ml by ethyl acetate and verbascoside (GI 0.28 and 0.09, respectively), and at 50 µg/ml by methanol extract and gedunin (GI 0.28 and 0.10, respectively). These parameters, together with the LD_{95} (the lethal dose producing 95% of death) values, established that the greatest effect was shown at 25.0 µg/ml by verbascoside (100% mortality), and at 35.0 µg/ml by ethyl acetate and methanol, respectively (100% mortality). Compared with the effect of a phytoecdysteroid such as ajugasterone C, which showed a GI of 0.88 at 100 µg/ml (Céspedes *et al.*, 2005), these extracts produced the highest insecticidal effects.

Table 15.6. Growth index (GI) and relative growth index (RGI) of *Spodoptera frugiperda* as a function of increased concentrations of extracts from *Calceolaria talcana*[a]

Compounds	Concentration (ppm)	GI[b]	RGI[c]
Control		0.95 ± 0.02a	1.0
Hexane	10.0	0.94 ± 0.12b	0.99
	20.0	0.93 ± 0.09b	0.98
	35.0	0.91 ± 0.08c	0.96
	50.0	0.71 ± 0.07d	0.75
Ethyl acetate	10.0	0.71 ± 0.08d	0.75
	20.0	0.41 ± 0.08e	0.45
	35.0	0.28 ± 0.05f	0.29
Methanol	10.0	0.93 ± 0.03b	0.98
	20.0	0.83 ± 0.08g	0.87
	35.0	0.69 ± 0.04d	0.73
	50.0	0.28 ± 0.04f	0.29
Verbascoside	10.0	0.66 ± 0.05h	0.67
	25.0	0.41 ± 0.03e	0.45
	35.0	0.09 ± 0.01i	0.15
	50.0	0.07 ± 0.01i	0.10
Gedunin[d]	10.0	0.77 ± 0.06d	0.71
	25.0	0.51 ± 0.04j	0.55
	50.0	0.10 ± 0.01k	0.11

[a]Mean of three replicates. [b]Means followed by the same letter within a column after ± standard error values are not significantly different in a Student-Newman-Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% confidence limits. [c]$RGI_{treatment} = GI_{treated} / GI_{control}$. [d]Data shown for gedunin are identical to those reported in Torres *et al.* (2003).

Interestingly, the most probable phytochemical composition of the active extracts of this study could be diterpenes, flavonoids, phenylethanoids, phenylpropanoids and iridoids (Di Fabio *et al.*, 1995; Garbarino *et al.*, 2000). Because these extracts have activity on moulting processes, it is possible that they can act in a similar manner to structures such as ecdysteroids. Its action is similar to juvenile hormone mimics that occur in higher plants. These extracts from Calceolariaceae species have similar activity to known juvenile hormone mimics but do not have the same structure composition. Insect growth regulatory activity on *S. frugiperda* was shown by phytoecdysteroids from *Ajuga remota* (Labiatae) (Kubo *et al.*, 1981) on two polyphagous (*Spodoptera littoralis* and *Ostrinia nubilalis*) and a monophagous (*Bombyx mori*) species (Marion-Poll and Descoins, 2002). Similar activities have been shown by ponasterone A, B and C and 20E, inokosterone and other terpenes that have been isolated from *Podocarpus nakaii* (Nakanishi *et al.*, 1966), from *P. elatus* (Galbraith and Horn, 1966), and from *P. nagi* and *P. gracilior* (Kubo *et al.*, 1993).

Although there is an ample body of literature about biological activities of phytoecdysteroids (Schmelz *et al.*, 1999; Dinan, 2001; Dinan *et al.*, 2001), there are no reports about insecticidal activity of any type of extracts from Calceolariaceae species; the only report about insecticidal activity from one *Calceolaria* is the insecticidal activity of a naphtoquinone isolated from *C. andina* (Khambay *et al.*, 1999).

15.5.8 Acute toxicity on last-stage larvae of *S. frugiperda*

To determine a possible correlation between IGR, acute toxicity and ecdysis caused by active extracts and verbascoside, the oral injection of 25 µg/ml of all samples into ten larvae (21 days old) of *S. frugiperda* was carried out. This concentration promoted apolysis to the fifth instar,

but inhibited ecdysis, whereas oral injection of 25 µg/ml of *n*-hexane resulted only in a delay of the normal moult to the fifth instar. Doubling the oral dose of verbascoside, ethyl acetate and methanol extracts to 50 µg/ml, after 48 and 72 h induced prothetely the appearance of precocious pupal structures in the larvae, in some (30%) of the treated fourth instar larvae. These larvae moulted directly to pupae. Prothetely can sometimes be elicited experimentally in larvae by the application of juvenile hormone or juvenile hormone mimics (Truman and Riddiford, 2002). Extract-induced prothetely resulted in precociousness and browning of pupae in roughly half of the controls.

15.5.9 Antioxidant activity of *C. talcana*

The antioxidant activity of extracts of *C. talcana* was evaluated using DPPH. All extracts showed some ability to sequester free radicals (Table 15.7, Fig. 15.3). The hexane extract showed a lower antioxidant capacity, reaching a percentage reduction of only 8.1% at concentrations as high as 300 ppm. The methanolic extract had an intermediate value of 41.35% activity at the same concentration. The aqueous extract, however, showed a percentage reduction of 97.74% to 47.46% at concentrations from 300 ppm to 50 ppm. The ethyl acetate extract showed the highest antioxidant capacity at the same concentrations, being the maximum of 97.58% at 300 ppm and 64.87% at 50 ppm.

15.5.10 *Condalia mycrophylla* Cav.

The milled sample from the aerial parts of *C. microphylla* was macerated with methanol and further partitioned with *n*-hexane, ethyl acetate and water. These extracts were used in a preliminary bioassay trial. Subsequently, in order to obtain more satisfactory data for insecticidal activity, some bioassays were carried out at lower concentrations. Gedunin and methanol extract from *Cedrela salvadorensis* (Me-Ced) were used as patterns and positive controls (Céspedes *et al.*, 2000, 2004, 2005; Torres *et al.*, 2003).

15.5.11 Phytochemical analysis

Table 15.8 shows the *n*-alkane composition (%) of *C. microphylla*. In the present work,

Table 15.7. Antioxidant activity of extracts from *Calceolaria talcana.*

Extracts	Concentration (ppm)	DPPH Reduction (%)*
n-Hexane	300	8.10 ± 0.72
	200	5.09 ± 0.45
	100	3.90 ± 0.34
	50	2.63 ± 0.23
Methanol	300	41.35 ± 3.67
	200	31.97 ± 2.83
	100	18.31 ± 1.62
	50	12.21 ± 1.08
Aqueous	300	97.74 ± 8.67
	200	97.11 ± 8.62
	100	59.45 ± 5.27
	50	47.46 ± 4.21
Ethyl acetate	300	97.58 ± 8.66
	200	97.05 ± 8.62
	100	69.27 ± 6.15
	50	64.87 ± 5.76

*DPPH Reduction %: (Blank absorbance - extract absorbance / blank absorbance) × 100. Each value corresponds to the average of the three different experiments ± SD. The significance level is p <95%.

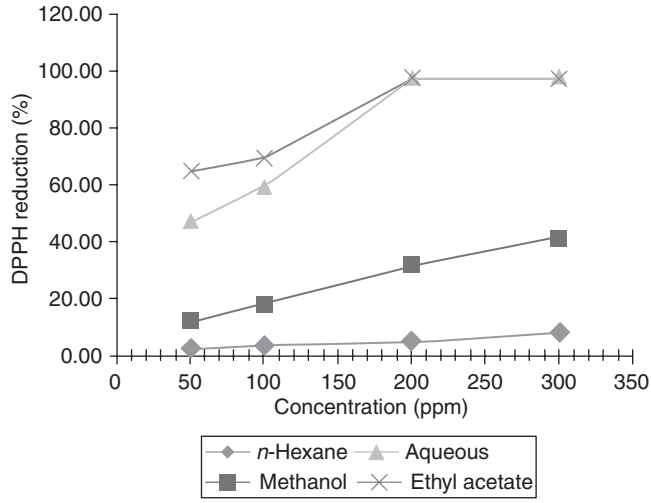

Fig. 15.3. Scavenging of DPPH radicals by extracts from *C. talcana*.

Table 15.8. Alkane composition (% total) of two populations of *Condalia microphylla* (collections 1 and 2) compared with two populations of the Cordoba Area, Argentina[a].

Compounds	Collection 1	Collection 2	Population 6[b]	Population 14[c]
Nonadecane (C-19)	4.0	4.8	4.9	3.2
Heinecosane (C-21)	2.2	2.0	2.1	2.0
Tricosane (C-23)	3.0	2.9	3.1	4.0
Pentacosane (C-25)	8.1	7.9	8.1	6.2
Heptacosane (C-27)	4.7	4.5	4.5	9.9
Octacosane (C-28)	3.8	3.8	5.5	6.3
Nonacosane (C-29)	31.2	29.0	26.0	30.0
Triacontane (C-30)	3.9	3.9	4.8	5.0
Hentriacontane (C-31)	33.7	32.7	34.3	12.4
Tritriacontane (C-33)	5.0	6.1	6.0	15.5
Unknown	0.4	2.4	0.7	5.5

[a]For populations 6 and 14, please see Zygadlo *et al.* (1992). [b]*C. microphylla* f. xanthocarpa (Cordoba area, Argentina). [c]*C. microphylla* f. melanocarpa (Cordoba area, Argentina).

the yield of *Condalia* neo-triacontanes and triacontanes (C-27, C-29, C-30, C-31 and C-33), using conventional extraction procedures (>35%), was comparable with literature data (Zygadlo *et al.*, 1992; Frontera *et al.*, 2000). On the other hand, *n*-hexane direct extraction was a little more efficient in its isolation from the plant (data not shown) than the conventional method with methanol. Interestingly, the yield of *n*-alkanes of the Chilean collection (collection 1) was slightly higher than that obtained for the Argentinian collection (collection 2). This phenomenon could be explained by the normal variation in the plant and by the phytogeographic characteristics of the collection sites, 'Montane grasslands and shrublands' in Argentina and 'Mediterranean forests woodlands and shrubs' in Chile, with the latter more arid than the former. The *n*-alkane composition determined in this work is similar to the composition of *Condalia* complex in the *n*-hexane extracts published by Zygadlo *et al.* (1992).

15.5.12 Antifeedant activity against *S. frugiperda*

The methanol, *n*-hexane and ethyl acetate extracts of *C. microphylla* showed a high feeding dissuasive activity against *S. frugiperda* larvae in the election assays (Table 15.9). The consumption of leaves treated with these extracts was significantly lower than in the controls and, in the case of the *n*-hexane extract, there were high levels of feeding inhibition, according to Hassanali and Lwande (1989). With the ethyl acetate extract, just a significant reduction of the consumption was observed; therefore, the effect of this extract, on the larvae can be considered as moderate. The methanol extract at 10 ppm showed a reduced to moderate effect (66%) and the most active extract was always *n*-hexane with 75% showing high deterrence power.

15.5.13 Insecticidal activity

Not-choice assays showed that three of four extracts possessed a lethal effect on the *T. molitor, S. frugiperda* and *D. melanogaster* larvae (Table 15.10). Against *S. frugiperda,* the extracts of *n*-hexane, ethyl acetate and methanol at 10 ppm showed 80, 70 and 70% of mortality effect, respectively. Against *T. molitor* at 10 ppm only *n*-hexane at 10 ppm showed a significant effect with a high percentage of mortality (73.5%). Against *D. melanogaster* the *n*-hexane extract showed the highest mortality percentage, with 94.5% at 10 ppm after 72 h. The same extract showed a mortality of 100% at concentrations above 25 ppm (Table 15.10).

Additionally, the effects of ethyl acetate, methanol and *n*-hexane extracts on growth and development of first instar larvae of *S. frugiperda* were evaluated initially at concentration of 2.0 ppm; the results are outlined in Table 15.11. Interestingly, methanol and ethyl acetate extracts induced a significant decrease in larval survival at 7 days. After 21 days the number of larvae and pupae decreased drastically in all treatments. When pupation did occur, serious abnormalities were observed, and this phenomenon was observed in similar form to *D. melanogaster*, where ecdysis and sclerotization were incomplete.

In not-choice experiments against first instar larvae of *S. frugiperda* and four instar larvae of *D. melanogaster* during the first 6 days, the effects of the ethyl acetate and methanol extracts were 100% lethal at concentrations greater than 50 ppm (data not shown). The concentrations that show 95% lethal doses (LD_{95}) of these extracts against *S. frugiperda* were: methanol (31.0 ppm), *n*-hexane (27.0 ppm) and ethyl acetate (42.0 ppm).

15.5.14 Insect growth inhibitory activity against *S. frugiperda* larvae

Intermediate concentrations (between 10.0 and 25.0 ppm) of extracts specifically

Table 15.9. Antifeedant inhibition (AI) obtained from antifeedant election tests, using different concentrations of extracts of *C. microphylla* on *S. frugiperda* larvae.

Concentration [ppm]	Control	Aqueous	Ethyl Acetate	*n*-Hexane	MeOH	Me-Ced	Gedunin
1	0	–5	5	5	13	5	21
5	0	–10	20	25	33	25	33
10	0	–15	40	75	66	37	45
25	0	–40	80	80	79	62	51
50	0	–51	85	98	90	89	89
100	0	–79	90	100	100	99	95

Results expressed as AI(%) = Antifeedant inhibition = [(1–T/C) × 100]. Control contains only solvent (Ethanol 15%); Me-Ced and gedunin were used as positive controls.

Table 15.10. Results obtained in tests with not-choice measured in mortality percentage of *S. frugiperda, T. molitor* and *D. melanogaster* larvae, after application of the extracts at different concentrations in the larvae's diet.

Sample	Concentration (ppm)	*S. frugiperda* (% mortality)	*T. molitor* (% mortality)	*D. melanogaster* (% mortality)	LD_{50} *S. frugiperda*	LD_{50} *T. molitor*	LD_{50} *D. melanogaster*
	Control	0	0	0.0			
Aqueous	10	0	0	0.0	n.d.	n.d.	n.d.
	25	0	0	0.0			
	50	0	0	0.0			
	100	0	0	0.0			
Ethyl acetate	10	70 ± 0.6b	45 ± 0.6b	61.0 ± 0.6a	9.4	14.2	7.65
	25	55 ± 0.7b	70 ± 0.7b	90.0 ± 0.6b			
	50	100 ± 0.8c	83.4 ± 0.8c	100.0 ± 1.0c			
	100	100 ± 1.0c	100 ± 1.0c	100.0 ± 1.0c			
Hexane	10	80 ± 0.2b	73.5 ± 0.3d	94.5 ± 0.6a	3.89	5.2	3.23
	25	90 ± 0.9c	84.7 ± 0.5b	100 ± 0.6a			
	50	100 ± 1.0c	95 ± 0.6b	100 ± 0.6b			
	100	100 ± 1.0c	100 ± 1.0c	100.0 ± 1.0c			
Methanol	10	70 ± 0.5b	40 ± 0.7b	30.0 ± 1.52	9.7	20.4	17.9
	25	50 ± 0.7b	55 ± 0.7b	65.0 ± 3.4b			
	50	100 ± 1.0c	80 ± 0.5b	100.0 ± 4.47c			
	100	100 ± 1.0c	100 ± 1.0c	100.0 ± 4.47c			
Me-Ced	10	54.0 ± 0.4d	25 ± 0.9a	70 ± 0.6a	48.0[a]		
	25	79.0 ± 0.3b	49 ± 0.8d	100 ± 1.0c			
	50	99.0 ± 0.9c	88 ± 0.7c	100 ± 1.0c			
Gedunin	10	37.0 ± 0.2a	22 ± 0.8a	69 ± 0.6a	10.8[a]		
	25	45.5 ± 0.3d	59 ± 0.9d	100 ± 1.0c			
	50	73.5 ± 0.6b	89 ± 0.4c	100 ± 1.0c			

Each value corresponds to the average of the five different experiments ± SE. The values followed by the same letter are not significantly different. The significance level is p <95%. The time for *S. frugiperda* was 21 days, for *T. molitor* was 25 days and for *D. melanogaster* was 72 h. [a]This value corresponds to LD_{95} (Céspedes *et al.*, 2005)

inhibited each larval growth stage, e.g. growth and weight gained (up to 75 % of length) when incorporated into diets (Table 15.11). Moreover, *n*-hexane extract produced the strongest inhibition (58.5% and 39.6% at 10.0 and 25.0 ppm, respectively) of growth and weight increase at 21 days (Table 15.11). On the other hand, the three extracts (*n*-hexane, ethyl acetate and methanol), above 25.0 ppm, showed a high growth inhibition, and after 21 days these extracts showed 100% of mortality, respectively (Table 15.11).

The percentage of larvae that reached pupation decreased drastically with almost all extracts assayed. Thus, *n*-hexane (10.0 ppm, 22.8%), ethyl acetate (10.0 ppm, 25.7%) and methanol (10 ppm, 26.3%) extracts showed a significant delay of pupation (Table 15.11). Above 50 ppm no larvae survived to pupation with *n*-hexane, ethyl acetate and methanol extracts (Table 15.11). Delays in time to pupation (>24 days) for *n*-hexane (>10.0 ppm), ethyl acetate (>15 ppm) and methanol (>35.0 ppm) were observed (data not shown). Furthermore, low concentrations of *n*-hexane, ethyl acetate and methanol extracts of between 2.0 and 10 ppm significantly reduced pupal weights, with *n*-hexane being the extract that produced the greatest effect on pupal weights between 1.0 to 5.0 ppm (data not shown).

The percentage of adults' emergence from the pupae was also drastically affected by these substances. The greatest reductions were shown by *n*-hexane (2.0 and 10 ppm, 91.7%), ethyl acetate (2.0 and 10 ppm, 83.4 and 91.7%, respectively) and methanol

Table 15.11. Activity of extracts from *C. microphylla* on pupation and emergence parameters of *S. frugiperda* (after 21 days of incubation)[a].

Treatment	Conc. (ppm)	Mean time pupation (days)[b]	Pupation SP (%)[e]	Mean weight pupae (mg)[c]	Mean emergence (days)[d]	Emergence (%)[f]	Male (%)	Female (%)
Control		22.0	88.2	309.5 ± 15.47a	33	77.50	35	42.5
n-hexane	2.0	22.0	60.6	190.5 ± 11.43a	31	8.3	8.3*	–
	10.0	22.5	22.8	180.9 ± 9.78b	33	8.3	8.3*	–
	25.0	24.0	16.8	122.7 ± 8.79b	–	0.0	–	–
	50.0	n.d.	0	n.d.	–	0.0	–	–
Ethyl acetate	2.0	21.5	68.3	227.6 ± 11.38a	33	16.7	8.3	8.3
	10.0	24.0	25.7	150.8 ± 7.54b	36	8.3	8.3*	–
	25.0	25.0	12.5	148.8 ± 7.44b	–	0.0	–	–
	50.0	25.0	6.3	n.d.	–	0.0	–	–
MeOH	2.0	22.0	52.3	205.3 ± 10.26a	32	16.6	8.3*	8.3
	10.0	25.0	26.3	119.9 ± 5.49b	35	8.3	8.3*	–
	25.0	25.0	6.3	109.0 ± 4.44b	–	–	–	–
	50.0	25.0	0	n.d.	–	–	–	–
Gedunin	10.0	22.5	49.8	111.5 ± 5.57b	34	16.6	8.3	8.3
	25.0	23.0	24.2	67.1 ± 3.35c	35	15.6	5.2	10.4
	50.0	24.0	4.17[d]	55.1 ± 2.75c	36	4.17	4.17*	–
Me-Ced	2.0	21.5	78.4	235.9 ± 1179a	33	33.3	11.1	22.2
	10.0	22.0	56.2	148.2 ± 7.41b	33	30.5	10.2	20.3
	25.0	23.5	29.5	124.3 ± 6.21b	34	20.8	10.4	10.4
	50.0	24.5	18.2	119.2 ± 5.54b	34	16.7	–	16.7

[a]The values for growth bioassay were from weight, values taken at 22 ± 1 days before pupation, the criteria followed were to account for larvae that formed pupae; the larvae that did not form pupae were counted as dead larvae. [b]Values taken after pupation. The values for aqueous extract were omitted because they are irrelevant, and this extract did not show any effect at all assayed concentrations. [c]Means followed by the same letter within a column after ± standard error values are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% confidence limits. [d]Percentage with respect to control. [e]SP: survival pupation = number of surviving pupae × 100/total larvae for pupation. [f]% = Number of adults emerged × 100/total number of pupae. The asterisks indicate deformities.

(2.0 and 10 ppm, 83.4 and 91.7%, respectively), and at these concentrations deformities could be also observed. Moreover, at concentrations between 2.0 ppm and 0.5 ppm this extract from *C. microphylla* significantly blocked the percentage of adult emergence, because no viable adults can emerge from pupae in this step.

In this study, analysis of the test insect fed with *C. microphylla* extracts revealed a developmental disruption in which the insects died (between 10 and 25 ppm) during pharate conditions after initiation of moulting (the apolysis step), without completion of morphogenesis. These last substances act in concert to trigger insect ecdysis during the final stages of the moult. Thus, the *n*-hexane extract of *C. microphylla* may disrupt several steps of ecdysteroid metabolism to result in an inhibition of emergence behaviour, or alternatively may act directly by inhibiting the release of ETH (Hesterlee and Morton, 1996). In our case, and based on toxicological information, the accumulation of long-chain *n*-alkanes as hentriacontane, for instance, by means of an unknown mechanism of action, could be affecting the complete morphogenesis of the insects used in our study. Because the hydrophobic environment prevents the action of enzymes, which require an aqueous medium for action (Jung and Deetz, 1993), all these considerations are supported under the evidence shown in Table 15.12, where

Table 15.12. Insect growth regulatory activity of the ethyl acetate, MeOH and *n*-hexane extracts from *C. microphylla*, Me-Ced and gedunin against *S. frugiperda* larvae in a not-choice bioassay[a].

	7 Days			21 Days		Pupation
Treatment	GWI_{50} [b]	GLI_{50} [c]	MC_{50} [d]	EI_{50} [b]	pI_{50} [e]	PI_{50} [f]
n-Hexane	8.6	5.3	3.9	0.55	0.26	3.46
Ethyl acetate	3.1	3.1	9.7	0.77	0.11	2.11
MeOH	4.0	8.4	3.5	3.10	0.49	4.62
Gedunin	2.7	5.9	27.9	0.66	0.18	9.96
Me-Ced	5.5	14.5	7.8	13.4	1.13	12.4

[a]The parameters in ppm values. [b]The GWI_{50} and EI_{50} correspond to the growth inhibition in weight at 7 and 21 days, respectively, and were calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA (p <0.05) under Microcal Origin 6.1. [c]GLI_{50} corresponds to the growth inhibition in length at 7 days and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA (p <0.05) under Microcal Origin 6.1. [d]MC_{50} is the concentration producing 50% mortality. [e]pI_{50} corresponds to –log EI_{50}. [f]PI_{50} corresponds to concentration producing 50% of pupation and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA (p <0.05) under Microcal Origin 6.1.

the pI_{50} values are very low for *n*-hexane (0.26) and ethyl acetate (0.11), for instance.

15.5.15 Growth inhibition and relative growth index for *S. frugiperda*

Larvae reaching the pupal stage in the groups of lowest concentration either do not pupate or emerge from pupae with deformities. Thus, in all treatments the average time to reach the mean weight of the adult stage relative to the time needed for control larvae to reach the adult stage was significantly delayed. The growth index (GI or number of surviving larvae/total larvae used) and relative growth index (RGI or $GI_{treated}$ /$GI_{control}$) (Table 15.13) show that the strongest effects are between 2.0 and 10.0 ppm by *n*-hexane extract (RGI 0.25), and at 25 ppm by methanol (RGI 0.25). These parameters, together with the LD_{95} (the lethal dose producing 95% of death) and LD_{50} values, established that the greatest effects were shown at 25.0 ppm by *n*-hexane (100% mortality), and by ethyl acetate and methanol at 35.0 ppm, respectively (100% mortality).

Interestingly, the phytochemical composition of the *n*-hexane extract of this study is mainly long-chain *n*-alkanes (Table 15.8) (Halse *et al.*, 1993; Delgado *et al.*, 2011). Because they have activity on morphological (moulting) processes, it is possible that they act in similar manner to structures such as ecdysteroids but with a new mode of action. Its action is similar to juvenile hormone mimics that occur in higher plants. However, this extract from *C. microphylla* with similar activity to known juvenile hormone mimics does not have exactly the same chemical structure as phytoecdysteroids. As mentioned, there is an ample body of literature about biological activities of phytoecdysteroids (Simon and Koolman, 1989; Sláma and Lafont, 1995; Schmelz *et al.*, 1999; Saez *et al.*, 2000; Savchenko, *et al.*, 2000; Dinan, 2001), but there are no reports about insecticidal activity of extracts from *C. microphylla* species with this type of activity, and this is the first report about insecticidal activity in this species.

15.5.16 Acute toxicity on last-stage larvae of *S. frugiperda* and *T. molitor*

In order to determine a possible correlation between IGR, acute toxicity and moulting disruption caused by these extracts, oral injections of 2, 10, 25 and 50 ppm of all extract samples into ten larvae of 21 days, of

Table 15.13. GI and RGI of *S. frugiperda* as a function of increased concentrations of ethyl acetate, MeOH and *n*-hexane extracts from *C. microphylla*, Me-Ced and gedunin.[a]

Compounds	Concentration (ppm)	GI[b]	RGI[c]
Control		0.99 ± 0.045[a]	
Me-Ced	2.0	0.99 ± 0.050b	1.00
	10.0	0.84 ± 0.085b	0.85
	25.0	0.75 ± 0.031b	0.75
	50.0	0.69 ± 0.055b	0.70
Ethyl acetate	2.0	0.75 ± 0.031b	0.75
	10.0	0.59 ± 0.040b	0.60
	25.0	0.25 ± 0.035c	0.25
	50.0	0.00	0.00
MeOH extract	2.0	0.99 ± 0.050b	1.00
	10.0	0.69 ± 0.055b	0.70
	25.0	0.25 ± 0.040b	0.25
	50.0	0.00	0.00
Hexane extract	2.0	0.25 ± 0.015c	0.25
	10.0	0.03 ± 0.015c	0.03
	25.0	0.00	0.00
	50.0	0.00	0.00
Gedunin	10.0	0.77 ± 0.060b	0.77
	25.0	0.51 ± 0.040b	0.51
	50.0	0.10 ± 0.010c	0.10

[a]Mean of three replicates. [b]Means followed by the same letter within a column after ± standard error values are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control). [c]$RGI_{treatment} = GI_{treated} / GI_{control}$.

S. frugiperda and of 25 days, of *T. molitor*, was carried out (Table 15.14). At 2.0 ppm of *n*-hexane extract apolysis was promoted to the fifth instar but moulting was inhibited, whereas oral injection at 10.0 ppm resulted only in a delay of the normal moult to the fifth instar. Increasing the oral dose of three extracts (*n*-hexane, ethyl acetate and methanol) to 10, 25 and 50 ppm induced the appearance of precocious pupal structures in the larvae (prothetely) (Marks, 1980), in some cases (>30%) of the treated fourth instar larvae. These larvae moulted directly to pupae. As mentioned, prothetely can sometimes be elicited experimentally in larvae by application of juvenile hormone or juvenile hormone mimics (Truman and Riddiford, 2002). Thus, *n*-hexane extract induced prothetely expressed in precociousness, browning and death of pupae and in a high adult mortality (data not shown). Consequently, *n*-hexane extract exhibited 100% larval mortality and gave the highest insecticidal activity.

15.5.17 Insect growth inhibitory activity against *T. molitor*

The *n*-hexane extract caused a strong decrease in the number of larvae of *T. molitor* that reach pupation (45% at 5.0 ppm), and ethyl acetate extract has this same effect without significant differences (39% at 2.0 ppm) (Table 15.15). With these extracts, as well as methanol, the larvae had a briefer time of pupation and emergence; however, many of the pupae were not viable and died (Table 15.15). At higher levels (>10 ppm) these extracts exhibited potent acute toxicity on larvae and pupae of *T. molitor*. In addition to a shorter pupal stage for those pupae that emerged, many pupae did not emerge. That effect was observed at 10, 25 and 10 ppm for *n*-hexane, methanol and ethyl acetate extracts, respectively (Table 15.15).

These results suggest that compounds in *n*-hexane and ethyl acetate extracts from *C. microphylla* have effects on

Table 15.14. Acute toxicity of ethyl acetate, MeOH and *n*-hexane extracts against larvae of last stage *S. frugiperda*.[a]

Compounds	Concentration (ppm)	% Survival[b] *S. frugiperda*	% Survival[b] *T. molitor*	LD_{50}[c] *S. frugiperda*	LD_{50} *T. molitor*
Control	0.0	100.0	100		
Ethyl acetate	2.0	79.5 ± 3.80b	91.5 ± 5.1a	7.99	13.74
	10.0	41.2 ± 2.00b	65.2 ± 3.9b		
	25.0	15.9 ± 0.85b	15.9 ± 0.77c		
	50.0	0	0		
n-Hexane	2.0	67.0 ± 3.70b	77.4 ± 2.9b	4.56	5.43
	10.0	30.0 ± 2.25b	27.3 ± 2.21c		
	25.0	7.0 ± 0.55a	10.1 ± 0.67d		
	50.0	0	0		
MeOH	2.0	90.9 ± 4.69b	90.0 ± 4.9a	24.0	24.2
	10.0	78.9 ± 3.95b	78.0 ± 3.8b		
	25.0	50.2 ± 3.51c	50.0 ± 3.3c		
	50.0	0	0		
Me-Ced	2.0	95.1 ± 4.75		36.65	
	10.0	78.1 ± 3.90			
	25.0	59.3 ± 2.96			
	50.0	45.0 ± 2.25			
Gedunin	10.0	54.7 ± 2.73b		10.78	
	25.0	14.1 ± 0.71c			

[a]After 24 h, survival of adults was recorded (percentage relative to controls). [b]Mean of three replicates. Means followed by the same letter within a column after ± standard error values are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control). [c]The LD_{50} is the lethal dose producing 50% survival.

ecdysone receptors (Dinan, 2001). From Table 15.8 it is possible to infer that *n*-hexane and ethyl acetate extracts accelerate the time of pupation for larvae of *T. molitor*. The *n*-hexane extract contains a high percentage of long-chain *n*-alkanes (Table 15.8) and exhibited acute toxicity to larvae of this insect with regard to the number of larvae that reached the pupal stage.

15.5.18 Remarks for *C. microphylla*

On the basis of these results, we suggest that the insect growth inhibition caused by *n*-hexane and ethyl acetate extracts from *C. microphylla* could be due to synergistic effects. These plant extracts may be considered to be efficient IGRs, as well as having activity similar to phytoecdysteroids, as was evidenced by their significant inhibition of moulting processes. These extracts had potent insecticidal and growth inhibitory activities. Probably the presence of long-chain *n*-alkane compounds in the *n*-hexane extract results in an increasing IGR activity. This finding suggests that these components could play an important role in both the insecticidal and IGR activity of *C. microphylla* extracts. The *n*-hexane and ethyl acetate extracts from *C. microphylla* have very good potency that was comparable to gedunin, Me-Yuc, Me-Myrt and Me-Ced previously reported (Céspedes *et al.*, 2000, 2004, 2005, 2006).

Preceding experimental observations suggest that acute toxicity and growth inhibition of our extracts may be due to inhibition of a proteinase, ETH and other polyphenol oxidases (PPO) that could be bound to these types of compounds or move throughout cellular membranes and could produce white substance accumulation (deposits) in vacuoles in similar form to that reported by Delgado *et al.* (2011) and Halse *et al.* (1993). This target has been demonstrated for other compounds of natural origin (Karban and Baxter, 2001; Kessler and Baldwin, 2002; Céspedes *et al.*, 2005).

Table 15.15. Growth inhibitory activities on *Tenebrio molitor* as a function of increased concentrations of extracts from *Condalia microphylla*.[a]

Samples	Doses (ppm)	Duration of pupal stages[b] 5 days	12 days	20 days	25 days	Number of pupae formed	Pupation (%)[c]	Successful emergence (%)[d]
Control		3	9	27	57	15.5 ± 0.4a	95	95
n-Hexane	2	5	25	39	43	14.5 ± 0.6a	73	5*
	5	11	23	28	30	10.9 ± 0.6b	45	5*
	10	10	25	26	27	n.d.	15	0
	25	5	12	17	20	n.d.	10	0
	50	3	10	11	12	n.d.	1	0
MeOH	2	5	25	40	45	16.1 ± 0.4b	76	5*
	5	11	30	40	46	14.5 ± 0.5a	79	5*
	10	12	25	27	30	n.d.	15	3*
	25	10	20	23	24	n.d.	13	0
	50	5	15	17	18	n.d.	5	0
Ethyl acetate	2	11	11	17	18	10.7 ± 0.6c	39	3*
	5	5	9	12	16	n.d.	20	1*
	10	3	9	13	17	n.d.	20	0
	25	3	10	15	18	n.d.	10	0
	50	3	5	7	10	n.d.	10	0
Me-Ced	10	10	15	20	32	15.9 ± 0.8a	25	5
	25	11	16	22	31	10.7 ± 0.6b	15	5
	50	7	12	19	22	9.0 ± 0.6b	10	3
Gedunin	10	7	15	22	29	9.2 ± 0.7b	3	0
	25	5	13	21	25	n.d	1	0
	50	3	11	20	24	n.d.	1	0

[a]Twenty larvae by assay and by triplicate, larvae of last stage, topical application. [b]Average duration, the criteria used were to measure until emergence of survival pupae. n.d. corresponds to pupae that do not produce any adult. Means followed by the same letter within a column after ± SE values are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% confidence limits. [c]Percentage with respect to control. [d]The asterisk indicate adults with deformities.

The sites and mode of action of these extracts and their isolated components are being investigated and probably correspond to a combination of antifeedant action as well as neurodegenerative effects, midgut phenol oxidase, proteinase, ETH, tyrosinase or other PPOs and cuticle synthesis inhibition, and moulting disruption and/or sclerotization toxicity, as has been found for other natural compounds (Céspedes *et al.*, 2000, 2004, 2005, 2006; Kubo *et al.*, 2003a,b; Torres *et al.*, 2003) and extracts (Feng *et al.*, 1995).

Thus, the effect of *n*-hexane, ethyl acetate and methanol extracts on reducing insect growth, increasing or shortening development time, modifying the apolysis during moulting and producing a high mortality on *T. molitor, S. frugiperda* and *D. melanogaster* were more powerful than gedunin and Me-Ced extract from *Cedrela salvadorensis* (Céspedes *et al.*, 2000, 2004, 2005, 2006; Torres *et al.*, 2003). Although chemically distinct, the level of insecticidal activity of metabolites and mixtures derived from this *Condalia* species is comparable to that of the known insect growth regulator, gedunin, and may be due to a synergistic effect shown by the ecdysone-like activity of the *n*-hexane extract in the test system used. Based on the present investigations, materials from Chilean Rhamnaceae should prove to be valuable sources of interesting biologically active compounds, including insecticides (Alarcon *et al.*, 2011; Céspedes *et al.*, 2011). New biological activity studies of the isolated compounds from these extracts are in progress.

15.5.19 Remarks for *C. talcana*

These results suggest that the effectiveness demonstrated by the ethyl acetate extract could be due to a synergistic effect of verbascoside in combination with another anti-insect compound(s) present in the extract or to the presence of a compound with higher activity than verbascoside. Although verbascoside showed effectiveness, its presence alone does not fully explain the activity of the extract. In order to answer this issue, we are currently conducting a bio-guided fractionation and the results will be published on completion.

These plant extracts may be considered to be efficient IGRs, as well as having similar activity to phytoecdysteroids, as was evidenced by their significant inhibition of moulting processes. They also had potent insecticidal and growth inhibitory activities, probably because the absence of polar compounds in the hexane extract results in a significant loss of its IGR activity, as was shown by the total phenolic content of polar extracts (Table 15.16; Céspedes *et al.*, 2005, 2006, 2009). The most active ethyl acetate extracts contain verbascoside as the major compound (a phenylethanoid glycoside). Ethyl acetate and methanol extracts had good potency that was comparable to gedunin, Me-Yuc, Me-Ced and Me-Myrt (Céspedes *et al.*, 2000, 2006, 2009; Torres *et al.*, 2003; Céspedes and Alarcon, 2011). These findings suggest that the ethyl acetate extract could play an important role in both the insecticidal and IGR activity of *C. talcana* extracts.

Table 15.16. Total phenolic content of *C. talcana* extracts.

Extract	Concentration
n-Hexane	144.3 ± 12.5a
Ethyl acetate	578.9 ± 19.6b
Methanol/water residue	355.0 ± 15.8c
Verbascoside*	72.3 mg/g extract

Values are expressed as μmol catechin equivalent/g extract. Values are the mean ± standard error from three replicates ($n = 3$). Different letters show significant differences at $p < 0.01$, using the Tukey test. *The concentration of verbascoside corresponds to mg of compound per g of extract.

Preceding experimental observations suggest that insecticidal and growth inhibition of our extracts may be due to inhibition of a proteinase, ETH and other polyphenol oxidases (PPO) that could bind to these compounds. These targets have been demonstrated for other compounds of natural origin (Kessler and Baldwin, 2002; Kubo *et al.*, 2003a,b; Céspedes *et al.*, 2005), together with other examples such as the insecticidal activity of extracts with similar content of phenylethanoids from *Stachys byzantine* (Asnaashari *et al.*, 2010) and the antioxidant and cholinesterase inhibition of *Verbascum xanthophoeniceum* (Georgiev *et al.*, 2011) among many other references of the literature. We are working in the elucidation and a dissection of the activities, sites and mechanisms of action of these extracts and compounds from *Calceolaria* species.

In summary, the insecticidal activity of our extracts from aerial parts from *C. talcana* may be due to a synergistic effect shown by the ecdysone-like activity of the extracts in the test system used in this investigation.

The sites and mode of action of these extracts continue to be investigated and probably correspond to a combination of antifeedant action, which is known at the molecular level that could have the following targets: midgut phenol oxidase, proteinase, ETH, tyrosinase or other PPOs and cuticle synthesis inhibition, as well as moulting sclerotization toxicity, as has been found for other natural compounds (Kubo 1997; Céspedes *et al.*, 2000, 2006; Kubo *et al.*, 2003a,b; Torres *et al.*, 2003; Céspedes and Alarcon, 2011) and extracts (Feng *et al.*, 1995).

15.6 Conclusions

The effect of verbascoside, ethyl acetate and methanol extracts on reducing insect growth, increasing or shortening development time, modifying the apolysis during

moulting and producing a high mortality on *S. frugiperda* and *D. melanogaster* were more powerful than gedunin, Me-Ced, Me-Myrt and Me-Yuc extracts (Céspedes *et al*., 2000, 2006; Torres *et al*., 2003). Although chemically distinct, the level of insecticidal activity of these extracts derived from this *Calceolariaceae* species is comparable to that of the known insect growth regulator, gedunin.

Finally, based on the present investigations, plant material from Chilean Calceolariaceae, Rhamnaceae and related species should prove to be valuable sources of new biologically active compounds, including insecticides. New biological activity studies of the isolated compounds are in progress.

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16 Anti-*Salmonella* Agents from the Brazilian Medicinal Plant *Tanacetum balsamita* and their Applications

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16.1 Introduction

The salmonellae are Gram-negative non-spore-forming rods. There are over 2500 serovars of *Salmonella*, all of which are presumed to be pathogenic to humans. Salmonellosis can result following the ingestion of viable cells of any member of the genus *Salmonella*. It is the second most common bacterial foodborne illness after *Campylobacter* infection. A search for anti-*Salmonella* agents was initiated by a request to solve the problem of pepper *Piper nigrum* (Piperaceae) fruit contamination by *Salmonella* in the Amazon basin. This contamination was probably caused by an increase in large-scale poultry farms around the areas where peppers are produced, because chickens are important sources of *Salmonella* contamination. *Salmonella* infection occurs when bacteria are ingested, typically from food derived from infected animals, but infection can also occur by ingesting the faeces of infected animals. Chickens may be infected with a number of different types of *Salmonella*, which then appear in the faecal matter (Guard-Petter, 2001). In addition, infected rodents and bats may contaminate unprotected pepper with their faeces and thereby spread *Salmonella* bacteria (Davies and Breslin, 2003). Currently, there are no appropriate anti-*Salmonella* agents available for pepper. Phytochemicals characterized as anti-*Salmonella* agents can be applicable in disinfecting and preventing the spread of these bacteria. For example, the anti-*Salmonella* phytochemicals may be mixed into artificial fodder, thereby reducing the presence of *Salmonella* in the faeces and consequently on the ground. Phytochemicals are different in structure from compounds of the better-studied microbial sources and hence their modes of action may very likely differ.

In our previous studies, some aliphatic 2*E*-alkenals characterized from a Brazilian medicinal plant, *Tanacetum balsamita* (Compositae), known locally as 'catinga de mulata', showed broad antimicrobial activity (Kubo and Kubo, 1995). Therefore, further evaluation of these 2*E*-alkenals against *Salmonella choleraesuis* may provide new insights into their antibacterial action on a molecular basis. The maximum antimicrobial activity of aliphatic 2*E*-alkenals is dependent on the balance of the hydrophobic alkyl (tail) chain length from the

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 Natural Antioxidants and Biocides from Wild Medicinal Plants (eds C.L. Céspedes *et al.*)

hydrophilic aldehyde group (head) (Kubo *et al.*, 1995a, 2003a). It is well known that the hydrophobicity of molecules is often associated with biological action (Hansch and Dunn, 1972). However, the rationale for this observation, especially the role of the hydrophobic portion itself, is still poorly understood and widely debated. Aliphatic 2*E*-alkenals represent an excellent model for investigating structure and activity relationships (SARs) related to this problem because these molecules possess the same hydrophilic portion (the enal group) but different hydrophobic alkyl portions. Thus, in addition to their potential as anti-*Salmonella* agents, an evaluation of these 2*E*-alkenals against *Salmonella* may provide new insights into the molecular basis of their antibacterial action. Aliphatic 2*E*-alkenals and their related analogues are common in many plants (Kubo and Kubo, 1995; Kubo *et al.*, 1996, 1999; Kubo and Fujita, 2001) and readily available. We tested a homologous series of aliphatic 2*E*-alkenals and their corresponding alkanals from C5 to C13, as well as a series of alkanols and other related compounds, for antibacterial activity against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640, one of the most frequent sources of bacterial food infections (Frazier and Westhoff, 1988).

16.2 2*E*-Alkenals

In previous reports, 2*E*-hexenal (C6) (**1**) (see Fig. 16.1 for structures) was described to show a broad antimicrobial spectrum (Kubo and Kubo, 1995; Kubo *et al.*, 1995a; Bisignano *et al.*, 2001; Gardini *et al.*, 2001; Lanciotti *et al.*, 2003), which includes activity against *S. choleraesuis, Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Proteus vulgaris* (Kubo and Fujita, 2001) and *S. choleraesuis*, as well as *Helicobacter pylori* (Kubo *et al.*, 1999). This aliphatic α,β-unsaturated aldehyde is known as 'leaf aldehyde' (Hatanaka, 1993)

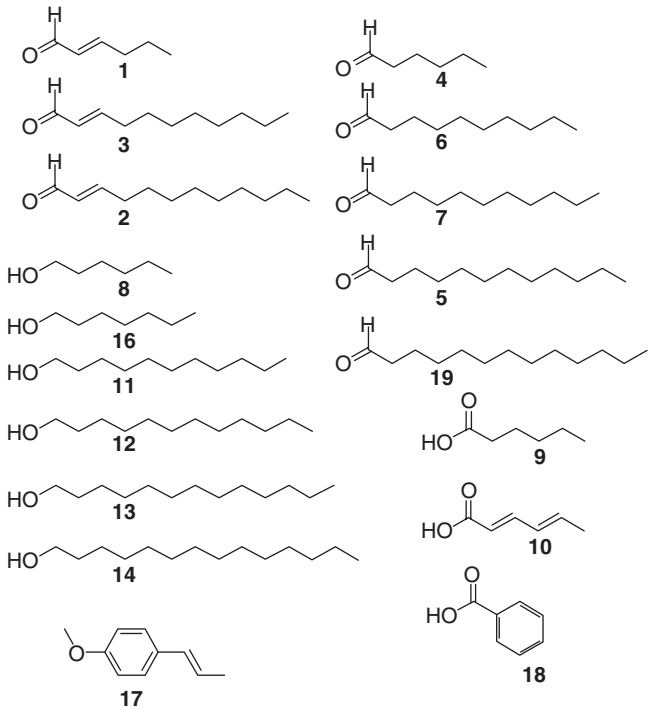

Fig. 16.1. 2*E*-Alkenals, alkanals, alkanols and related compounds.

and is widely distributed in many plants (Schauenstein, 1977). In our continuing search for antimicrobial agents from edible plants, 2*E*-hexenal was previously characterized as an antimicrobial agent from the volatile fraction of the cashew apple (Muroi *et al.*, 1993), coriander and olive oil (Kubo *et al.*, 1995a; Bisignano *et al.*, 2001). The bactericidal effect of 2*E*-hexenal was confirmed by the time kill curve experiment as shown in Fig. 16.2. Cultures of *S. choleraesuis*, with a cell density of 1×10^5 CFU/ml, were exposed to two different concentrations of 2*E*-hexenal. The number of viable cells was determined following different periods of incubation with 2*E*-hexenal. The result verifies that minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are the same. In contrast to 2*E*-hexenal, hexanol did not show any activity against *S. choleraesuis* up to 1600 μg/ml, but hexanal and hexanoic acid still exhibited some activity, albeit to a lesser extent than 2*E*-hexenal. Thus the conjugated double bond is not essential to elicit the antibacterial activity but is associated with increasing the activity.

The maximum antimicrobial activity of 2*E*-alkenals is dependent on the balance of the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head) (Kubo *et al.*, 1995a, 2003a). It is well known that the hydrophobicity of molecules is often associated with biological action (Hansch and Dunn, 1972). However, the rationale for this observation, especially the role of the hydrophobic portion, is still poorly understood and widely debated. To clarify this, 2*E*-alkenals are a superior model for structure and anti-*Salmonella* activity relationship (SAR) study because these molecules possess the same hydrophilic portion, the enal group, which explains the role of the hydrophobic alkyl portion.

A homologous series of aliphatic 2*E*-alkenals and their related analogues are common in many plants and readily available. Therefore, a homologous series of aliphatic 2*E*-alkenals, as well as the corresponding alkanals, from C5 to C13 were tested for their antibacterial activity against *S. choleraesuis* using a twofold broth dilution method for comparison. The results are listed in Table 16.1. The range of the antibacterial activity of the 2*E*-alkenals tested against *S. choleraesuis* is between 6.25 and 200 μg/ml, and the MICs and MBCs are markedly the same. The antibacterial activity against this foodborne bacterium is

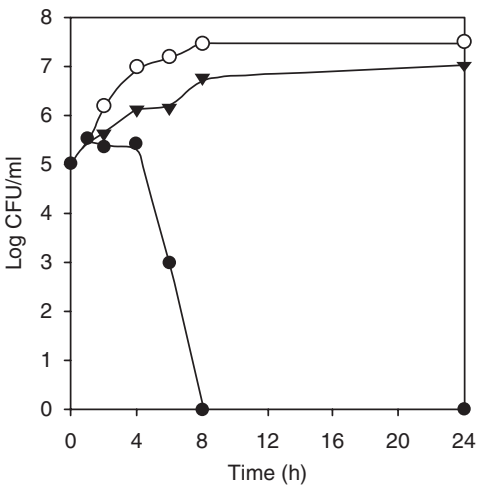

Fig. 16.2. Bactericidal effect of 2*E*-hexenal against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640. Exponentially growing cells of *S. choleraesuis* were inoculated at 37°C in nutrient yeast glucose (NYG) broth with 0 (○), 50 (▼) or 100 (●) μg/ml of 2*E*-hexenal. Viability was established by the number of colonies formed on NYG plate after incubation at 30°C for 24 h.

Table 16.1. Antibacterial activity (μg/ml) of aliphatic aldehyde compounds against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640.

Aldehydes tested	2*E*-Alkenal		Alkanal	
	MIC	MBC	MIC	MBC
C_5	200	200	–	–
C_6	100	100	400	800
C_7	100	100	400	400
C_8	100	100	200	400
C_9	50	50	100	200
C_{10}	25	25	100	100
C_{11}	12.5	12.5	100	100
C_{12}	6.25	6.25	100	100
C_{13}	25	200	>800	>800

–, Not tested.

correlated with the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head). Among the compounds tested, 2*E*-dodecenal (**2**) was found to possess the most potent activity against *S. choleraesuis*, followed by 2*E*-undecenal (**3**). 2*E*-Dodecenal exhibited the activity with both MIC and MBC of 6.25 μg/ml (34 μM), suggesting that no residual bacteriostatic activity is involved. Notably, this MBC value is slightly more potent than that of gentamicin. It appears that *S. choleraesuis* showed different susceptibilities to aldehydes possessing different chain lengths. This result is broadly similar to those of the corresponding alkanols against many microorganisms (Kubo *et al.*, 1995b, 2003b), indicating at least in part the similarity of their mode of action. Because of their easy availability and broad antimicrobial activity, 2*E*-alkenals, 2*E*-hexenal and 2*E*-dodecenal were further studied in detail.

The bactericidal effect of 2*E*-dodecenal was confirmed by the time kill curve method as shown in Fig. 16.3. Cultures of *S. choleraesuis*, with a cell density of 10^5 CFU/ml, were exposed to three different concentrations

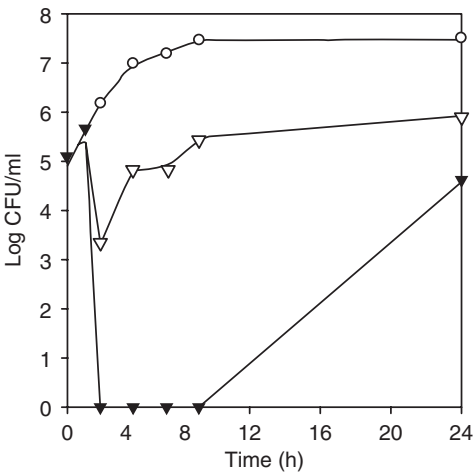

Fig. 16.3. Effect of 2*E*-dodecenal on the growth of *S. choleraesuis* subsp. *choleraesuis* ATCC 35640. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. The arrow indicates the time when drug was added. 2*E*-Dodecenal 0 (○), 1.56 (▽), 3.13 (▼) and 6.25 (●) μg/ml.

of 2*E*-dodecenal. The number of viable cells was determined following different periods of incubation with 2*E*-dodecenal. The result verifies that the MIC and MBC are the same. Notably, lethality occurred quickly, within the first 1 h after the addition of 2*E*-dodecenal. This rapid lethality very likely indicates that antibacterial activity of 2*E*-dodecenal against *S. choleraesuis* is associated at least in part with physicochemical damage to the cells, such as the disruption of the membrane.

The bactericidal effect of 2*E*-hexenal occurred slower than that of 2*E*-dodecenal, which needed 7 h. Such a slow cell death is thought to proceed independently of the membrane disruptive action. The result obtained indicates that the mode of antibacterial action of 2*E*-hexenal and 2*E*-dodecenal against *S. choleraesuis* differs to some extent. The effects of 2*E*-dodecenal and 2*E*-hexenal against *S. choleraesuis* were further tested by holding the viable cell number in the presence of chloramphenicol. This antibiotic is known to restrict cell division by inhibiting protein synthesis. Figure 16.4 shows that the effect of chloramphenicol against *S. choleraesuis* cells is bacteriostatic for the first 3 h after the addition of the drug. It should be noted that chloramphenicol is known to be bacteriostatic for a wide range of Gram-positive and Gram-negative bacteria, but this antibiotic expressed a bactericidal effect against *S. choleraesuis* after 8 h incubation. In the presence of chloramphenicol, 2*E*-hexenal decreased viable cell numbers slightly more quickly than in the absence. 2*E*-Dodecenal induced rapid decrease in viability regardless of the presence of chloramphenicol. The inhibition of cell division by chloramphenicol did not influence the bactericidal effects of 2*E*-hexenal and 2*E*-dodecenal. The reduced viability might not be due to interaction with the biosynthesis of cell wall or plasma membrane components. The synthesis of macromolecules such as DNA, RNA and proteins was not related to the reduction. The observation of the rapid bactericidal effect of 2*E*-dodecenal very likely indicates that the primary action of 2*E*-dodecenal is on the cell membrane.

In addition to the surfactant concept, 2*E*-alkenals may enter into the cells by passive diffusion across the plasma membrane. It also probably permeates in part through pores derived from membrane damage. If this is so, the α,β-unsaturated aldehyde group should not be overlooked because this group is chemically highly reactive and readily reacts with biologically important nucleophilic groups, such as sulfhydryl, amino or hydroxyl. Once inside the cells, 2*E*-alkenals may react with various intercellular components, for example, sulfhydryl groups in proteins and lower molecular weight compounds. It is known that α,β-unsaturated aldehyde group reacts with sulfhydryl groups mainly by 1,4-additions under physiological conditions (Schauenstein *et al.*, 1977). 2*E*-Alkenals were reported to cause depletion of cytoplasmic and mitochondrial glutathione, which functions in eliminating reactive oxygen species (Machida *et al.*, 1998). Sulfhydryl groups in proteins and lower molecular weight compounds such as glutathione are known to play an important role in the living cell. Bacteria protect themselves against hydrogen peroxide in various ways (Brul and Coote, 1999), and some of the most ubiquitous systems include glutathione. Taking these factors altogether, 2*E*-alkenals first act as a surfactant and then inhibit various cellular functions non-specifically, and thus 2*E*-alkenals do not act by a single defined process but have multiple functions, depending on their alkyl chain length.

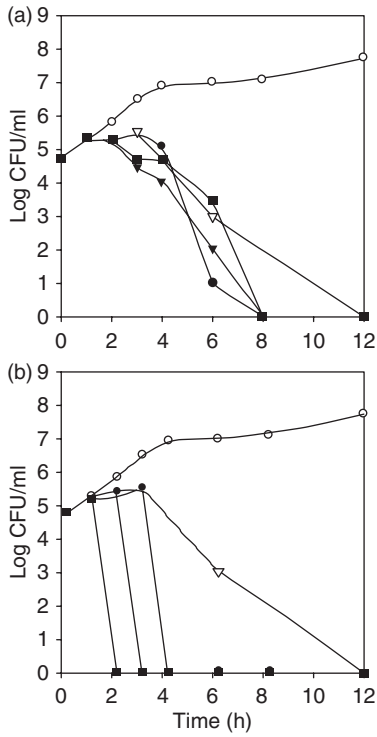

Fig. 16.4. Effect of (a) 2*E*-hexenal and (b) 2*E*-dodecenal in the presence of chloramphenicol against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. Chloramphenicol 0 (○); and 6.25 (▽) μg/ml was added to the culture after 1 h cultivation. 2*E*-Hexenal (100 μg/ml) or (2*E*)-dodecenal (6.25 μg/ml) and chloramphenicol (6.25 μg/ml) were added at 1 (■), 2 (▼) and 3 (●) h.

Subsequently, hexanal (C6) (**4**) was also found to exhibit the antibacterial activity against *S. choleraesuis* with MIC and MBC of 400 and 800 μg/ml, respectively. It seems that the antibacterial activity against *S. choleraesuis* should not be specific to 2*E*-alkenals because the conjugated double bond is not essential in eliciting activity, but is involved with increasing the activity. This prompted us to test the corresponding alkanals for their antibacterial activity against *S. choleraesuis* for comparison. The results are listed in Table 16.1. The activity of alkanals is weaker than those of the corresponding 2*E*-alkenals. Similar to 2*E*-alkenals, their MIC and MBC values are approximately the same and the activity also increased in general with increasing carbon chain length up to dodecanal (C12) (**5**). It should be noted, however, that there is a slight difference between 2*E*-alkenals and alkanals. For example, decanal (C10) (**6**), undecanal (C11) (**7**) and dodecanal are the most effective, but their MIC and MBC values against *S. choleraesuis* are all the same. The increase in the activity as carbon-chain length increases is not distinct in the case of alkanals as compared to those of 2*E*-alkenals. The bactericidal effect of hexanal and dodecanal were also confirmed by the time kill curve method (data not illustrated). In contrast to 2*E*-hexenal, hexanol (**8**) did not show any activity against *S. choleraesuis* up to 1600 μg/ml as listed in Table 16.2. Both

Table 16.2. Antibacterial activity (μg/ml) of the selected compounds against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640.

Compounds tested	MIC	MBC
2*E*-Hexenal	100	100
Hexanal	400	800
Hexanol	>1600	>1600
Hexanoic acid	400	400
Sorbic acid	400	400
Gentamicin	12.5	12.5

hexanoic acid (**9**) and sorbic acid (**10**) exhibited the same weak activity, indicating that the conjugated double bond in **10** is unlikely to be related to the activity.

Although 2*E*-hexenal is not the most effective against *S. choleraesuis*, it was selected to disinfect the contaminated pepper because of its high volatility (Wilson and Winiewski, 1989) and broad antimicrobial spectrum (Kubo *et al.*, 1995a; Lanciotti *et al.*, 2003). In addition, 2*E*-hexenal is the predominant volatile component that has been found in vegetative portions of virtually all plant species (Hatanaka, 1993) and was previously reported to be negative for a mutagenicity test (Andersen *et al.*, 1994). The pepper is washed with 0.1% of 2*E*-hexenal in ethanol for 3 min before the final drying process. It may also be worthwhile considering the anti-*Salmonella* activity of rather common phytochemicals from an ecological point of view. For example, it should be remembered that chickens used to peck green leaves. The green leaves contain a variety of antibacterial agents against *Salmonella* bacteria, especially those known as green leaf aldehydes and alcohols (Hatanaka, 1993). This indicates that *Salmonella* spp. are probably controlled in nature when chickens were continuously fed green-leaf-based food.

16.3 Alkanols

2*E*-Alkenals are highly reactive chemicals, which may limit their practical use. This prompted us to test a series of aliphatic primary alcohols (hereafter referred to simply as alkanols) from C5 to C14 for antibacterial activity against the same *S. choleraesuis* strain. The results are listed in Table 16.3. The range of the antibacterial activity of the alkanols tested against this foodborne bacterium is between 6.25 and 800 μg/ml, and MICs and MBCs are markedly similar to each other. Dodecanol was found to be the most effective against *S. choleraesuis* with an MBC of 6.25 μg/ml (34 μM), followed by undecanol (C11) (**11**) with an MBC of 12.5 μg/ml (73 μM). This MBC of dodecanol (C12) (**12**) is slightly more potent than that of gentamicin. It seems that *S. choleraesuis* showed different susceptibility to alkanols possessing different chain length but, in agreement with many other studies of the homologous series of alkanols, the antibacterial activity of the alkanols against *S. choleraesuis* increased with number of carbons in the chain until reaching the MIC and MBC, in this experiment at dodecanol and undecanol, respectively. Noticeably, the activity disappears after the chain length reached the MIC and MBC, known as the so-called 'cutoff' phenomenon (Balgavy and Devínsky, 1996). For example, dodecanol was found to be the most effective with the MIC and MBC of 6.25 μg/ml, and the MIC and MBC of tridecanol (C13) (**13**) are almost the same as those of dodecanol but they were variable. Tetradecanol (C14) (**14**) no

Table 16.3. Antibacterial activity (μg/ml) of alkanols against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640.

Alkanols tested	MIC	MBC
C_5	>1600	>1600
C_6	>1600	>1600
C_7	800	800
C_8	400	400
C_9	200	200
C_{10}	50	50
C_{11}	12.5	12.5
C_{12}	6.25	6.25
C_{13}	6.25–50*	6.25–100*
C_{14}	>100	>100

*The values are variable.

longer showed any activity up to 100 µg/ml. In other words, dodecanol is the most effective. On the other hand, hexanol (C6) did not exhibit any activity against *S. choleraesuis* up to 1600 µg/ml. It appears that *S. choleraesuis* showed different susceptibility to alkanols possessing different chain length. The similar parabolic function of the lipophilicity and maximized with alkyl chain lengths was previously reported with a series of alkyldimethylbenzylammonium chlorides (Daoud *et al.*, 1983). Notably, no alkanol exhibits any noticeable antibacterial activity against the other Gram-negative bacteria tested: *E. coli, P. aeruginosa, E. aerogenes, P. vulgaris* and *H. pylori*. In other words, alkanols fall short of the broad spectrum of activity as far as Gram-negative bacteria are concerned but specifically against *S. choleraesuis*. It seems that *S. choleraesuis* differs from other Gram-negative bacteria. This difference may be caused by their different permeability of the outer membrane layer because this plays a major role in the general resistance of Gram-negative bacteria, especially to lipophilic antibiotics. Noticeably, most Gram-negative bacteria are surrounded by the outer membrane and this functions as an effective but less specific barrier (Nikaido, 1994). If the selective elimination of *Salmonella* bacteria is desirable, alkanols may be considered to be superior.

The bactericidal effect of decanol against *S. choleraesuis* was confirmed by the time kill curve method as shown in Fig. 16.5. Cultures of *S. choleraesuis*, with a cell density of 5×10^5 CFU/ml, were exposed to two different concentrations of decanol. The number of viable cells was determined following different periods of incubation with decanol. The result verifies that MIC and MBC are the same. It shows that ½MIC slowed growth but that the final cell count was not significantly different from the control. The result shows that lethality occurred notably quickly, within the first 1 h after the addition of decanol. This rapid lethality very likely indicates that the antibacterial activity of decanol against *S. choleraesuis* is associated with the disruption of the membrane, similar to the effect described against *Saccharomyces cerevisiae* (Kubo *et al.*, 1992; Kubo and Fujita, 2001).

Similarly, the bactericidal effect of dodecanol was also confirmed by the time kill curve method as shown in Fig. 16.6.

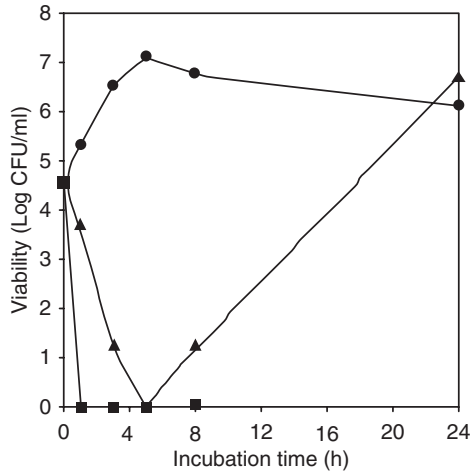

Fig. 16.5. Effect of decanol on the growth of *S. choleraesuis* subsp. *choleraesuis* ATCC 35640. Exponentially growing cells were inoculated into NYG broth and cultured at 37°C. Decanol, 0 (●), 25 (▲), and 50 (■) µg/ml.

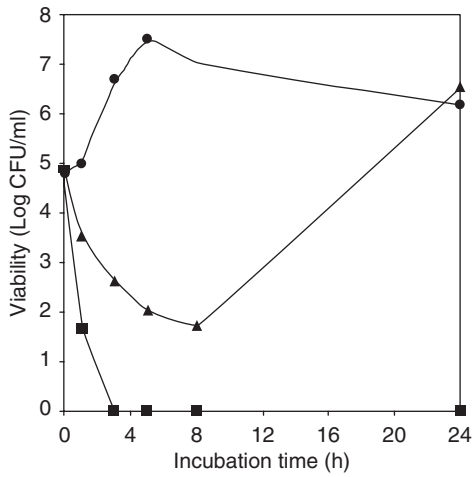

Fig. 16.6. Effect of dodecanol on the growth of *S. choleraesuis* subsp. *choleraesuis* ATCC 35640. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. Dodecanol, 0 (●), 3.13 (▲), and 6.25 (■) µg/ml.

Cultures of *S. choleraesuis*, with a cell density of 5×10^5 CFU/ml, were exposed to two different concentrations of dodecanol. The number of viable cells was determined following different periods of incubation with dodecanol. The result verifies that MIC and MBC are the same. It shows that ½MIC slowed growth but the final cell count was not significantly different from the control. In the time kill curve experiment, lethality occurred notably quickly, within the first 1 h after the addition of dodecanol. Similar to that found for decanol, the antibacterial activity of dodecanol is also probably linked to the membrane disruptive effect.

The effect of dodecanol on the growth of *S. choleraesuis* treated with chloramphenicol was examined. Cell viability of *S. choleraesuis* in the presence of 6.25 μg/ml of chloramphenicol was kept at the same level during incubation, as shown in Fig. 16.7. Dodecanol reduced the viability rapidly regardless of the treatment of chloramphenicol. This antibiotic is known to inhibit transpeptidation in protein synthesis, thereby restricting cell division. Hence, the bactericidal effect of dodecanol (**12**) is not thought to be the necessary function accompanying reproduction of *S. choleraesuis* cells, which involves macromolecule biosyntheses such as DNA, RNA and protein, and cell wall synthesis.

The effect of the growth phase on the bactericidal ability of dodecanol against *S. choleraesuis* was investigated (Fig. 16.8). After 2- and 5-h incubation MBC of dodecanol was added to the culture of *S. choleraesuis*. It reduced cell viability rapidly within 1 or 2 h following addition. After 5 h its exponential cell growth had stopped, representing entry into a stationary phase. This result indicates that dodecanol acts regardless of the growth phase, supporting bactericidal action against non-growing cells as described above.

Subsequently, the bactericidal activity of heptanol (C7) (**16**) was also confirmed by time kill experiment. The lethality needed 7 h and occurred slower than that of dodecanol (data not illustrated). Such a slow cell death is thought to proceed independently of the membrane disruptive action. Notably,

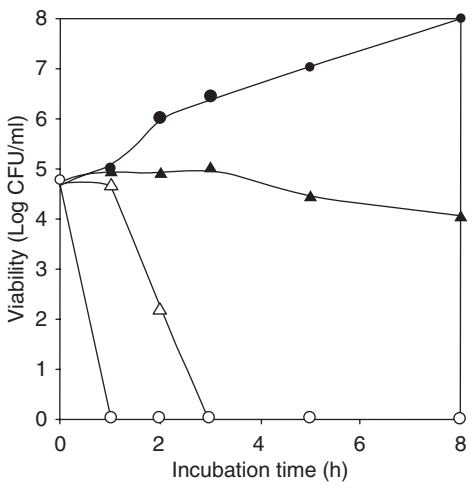

Fig. 16.7. Effect of dodecanol on the growth of *S. choleraesuis* subsp. *choleraesuis* in the presence of chloramphenicol. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. Chloramphenicol at 0 (●, ○), and 6.25 (▲, △) μg/ml was added to each culture at 0 h. Dodecanol at 12.5 μg/ml was added at 0 (○) and 1 (△) h.

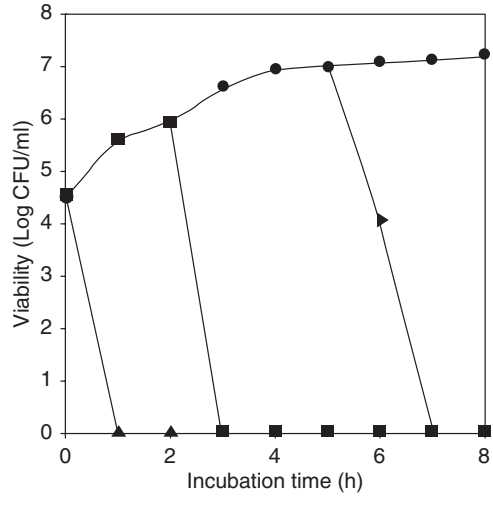

Fig. 16.8. Effect of growth phase on bactericidal activity of dodecanol against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640. The exponentially growing cells of *S. choleraesuis* were incubated in NYG broth at 37°C. Control (●) indicates incubation without dodecanol. Dodecanol (6.25 μg/mL) was added to the culture at 0 (▲), 2 (■), and 5 (♦) h.

heptanol inhibited only actively growing and dividing *S. choleraesuis* cells. Hence, there seem to be other factors involved to explain the antibacterial action of short-chain (C8) alkanols, but the rationale still remains unknown.

Combining two or more antimicrobial compounds seems to be superior to enhance and/or broaden the total activity. In previous papers, a common phenylpropanoid, anethole (**17**), was described to enhance the antifungal activity of 2*E*-undecenal (**3**) against *S. cerevisiae* (Kubo and Kubo, 1995). Hence, dodecanol was combined with anethole to see if the combination has any enhancing activity against *S. choleraesuis*. The combination of anethole synergistically retarded the growth rate of this foodborne bacterium to a large extent, but the combination was not bactericidal. Its bactericidal action was only marginally synergistic. In connection with this, anethole itself exhibited antibacterial activity against *S. choleraesuis* with both MIC and MBC of 200 μg/ml (1.35 mM). No differences in its MIC and MBC were noted, suggesting that the activity is bactericidal. The bactericidal effect of anethole against *S. choleraesuis* was confirmed by the time kill curve method (Kubo *et al.*, 1995b; Fujita *et al*, 2007). The reason for the residual bacteriostatic activity of the combination against *S. choleraesuis* remains unknown.

In our previous studies on structure–antifungal activity relationships with a series of primary alkanols, we reported that the antifungal activity of medium chain (C9–C12) alkanols against *S. cerevisiae* was mediated primarily owing to their non-ionic surface-active properties disrupting the lipid–protein interface non-specifically, and the maximum activity can be obtained when balance between the hydrophilic and hydrophobic portions becomes the most appropriate (Kubo *et al.*, 1995b, 2003b). This surfactant concept can also be applicable in part against *S. choleraesuis*, because in the time kill experiment: (i) lethality occurred notably quickly within the first 1 h after the addition of one of the medium-chain alkanols; (ii) bactericidal activity was found at any growth stage; and (iii) dodecanol rapidly killed *S. choleraesuis* cells in which cell division was inhibited by chloramphenicol. Moreover, the antimicrobial activity of alkanols is non-specific and the potency of the activity against *S. choleraesuis* was distinctly increased with each additional CH_2 group, up to dodecanol. The observed results support medium chain alkanols' ability to function as non-ionic surfactants.

As non-ionic surfactants, alkanols first approach the binding site with the electron negativity of the hydroxyl oxygen atom. This hydrogen bond acceptor will affect the hydrogen bonds that regulate the permeability of the lipid bilayers. For example, the hydroxyl group of cholesterol resides near the membrane–water interface in the lipid bilayers and is likely to be bonded with the carbonyl group of phospholipids (Brockerhoff, 1974; Chauhan *et al.*, 1984; Chiou *et al.*, 1990). Alkanols may function by disrupting and disorganizing the hydrogen bonds. Cholesterol is a major component of the animal plasma membrane and owes its membrane-closing properties to its rigid longitudinal orientation in the membrane. Cholesterol has profound influences on membrane structure and function; therefore, if the hydrogen bond is broken, cell function will be impaired.

Given the surfactant-like properties of medium-chain (C9–C12) alkanols, it is possible to suggest that alkanols also act at the lipid–protein interface of integral proteins, such as ion channels and/or transport proteins, denaturing their functioning conformation in a similar manner as that described against *S. cerevisiae* (Heidmann *et al.*, 1983; Nikaido, 1994). The common nature among these alkanols should be considered in that the electron negativity on the hydroxyl oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be disturbed maximally by hydrophobic compounds of particular hydrophilic hydroxyl group. Thus, the medium-chain alkanols disrupt the hydrogen bonding in the lipid–protein interface in *S. choleraesuis*. The data obtained are

consistent with an effect on the bulk membrane rather than a direct interaction of the specific target protein, and the alkanols' non-specificity of antimicrobial activity supports this assumption. The possibility of anti-*Salmonella* activity of the medium-chain alkanols is due to their non-ionic surfactant property, but this may not be the case for short-chain alkanols. The short-chain alkanols enter the cell by passive diffusion across the plasma membrane and/or through porin channels (Schulz, 1996). On the other hand, the more lipophilic long-chain alkanol molecules, being dissolved in the medium, are incorporated in part into the lipid bilayers (Franks and Lieb, 1986). The amount of alkanols entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. None the less, alkanols are chemically stable compounds and may not react with any biologically important substances in the cytosol or lipid bilayer. Hence, the primary antibacterial action of medium-chain alkanols comes from their ability to function as non-ionic surfactants (physical disruption of the membrane). This may reveal the different effects of alkanols on *S. choleraesuis* as compared to the corresponding 2*E*-alkenals; that is, α,β-unsaturated aldehydes are chemically highly reactive substances and hence 2*E*-alkenals, being entered into the cytosol or lipid bilayer, may readily react with biologically important nucleophilic groups, such as sulfhydryl, amino or hydroxyl groups (Schauenstein *et al.*, 1977). For example, hexanol did not exhibit any activity against *S. choleraesuis* up to 1600 μg/ml, whereas 2*E*-hexenal showed the bactericidal activity with a MBC of 100 μg/ml (Kubo and Kubo, 1995; Bisignano *et al.*, 2001; Kubo and Fujita, 2001). It appears that medium-chain 2*E*-alkenals first act as surfactants and are then involved in biochemical processes.

The same series of alkanols were recently found to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver. The potency increased with increasing chain length up to undecanol. Given each alkanol's nearly identical effect on State 3 and uncoupled respiration, this action is not directly on the ATP synthetase but earlier in the respiratory process. Hexanol and decanol were assayed against freeze–thawed (broken) mitochondria to distinguish effects on the mitochondrial substrate carrier from that on the electron transport chain. Both alcohols were only weak inhibitors of respiration in broken mitochondria, suggesting that inhibition originates from interference with the dicarboxylate carrier that must transport succinate across the mitochondrial membranes. Alkanols may inhibit this transporter in the inner membrane as non-ionic surfactants. The results with mitochondria also support the alkanols' non-ionic surfactant concept because enzyme systems related to transport of solutes and electron transfer are located in the inner membrane of the cell envelope of *Salmonella* (Hammond and Kubo, 2000). On the other hand, alkanols with longer carbon-chain length were previously reported to exert a stronger inhibitory effect on the $(Na^+ + K^+)$-ATPase activity of the neural membranes (Sun and Samorajski, 1975), probably by a similar surfactant concept to that described for H^+-ATPase (Nikaido, 1994). Overall, it seems that the anti-*Salmonella* activity of alkanols is mediated by biophysical processes. In addition, an increase in the alkyl chain length results in a parallel increase in the surface tension (Leshem *et al.*, 1988). Surface tension changes may be triggered in another purely biophysical manner, which needs to be considered. For example, surface tension could affect mobility and/or exposure of membrane-embedded proteins such as enzymes and receptors. The data obtained indicate, however, that the effect of surface tension is not primarily related to the activity (Bisignano *et al.*, 2001) but cannot be entirely ruled out.

In addition, the antibacterial activity of the homologous series of alkanals against *S. choleraesuis* was also tested for comparison. The results are listed in Table 16.2. Notably, the potency of the activity against this foodborne bacterium was not increased for each additional CH_2 group. Similar to what is found for alkanols, the short-chain alkenals enter the cell by passive diffusion

across the plasma membrane and/or through porin channels (Schulz, 1996), and long-chain alkanals that are dissolved in the medium are incorporated into the plasma membrane lipid bilayers (Franks and Lieb, 1986). The amount of alkanals entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. Once inside the cytosol or lipid bilayer, alkanals may react with biologically important substances, because aldehydes are chemically reactive compounds. Tridecanal is inactive because it may not attain high enough concentrations in the lipid bilayer. It appears that the bactericidal action of alkanals is similar to those described for alkanols in many aspects but differs to some extent.

Alcohols are among the most versatile of all organic compounds, and free and esterified alcohols are known to occur widely in nature. Safety is a primary consideration for chemical preservatives, especially concerning their use in food products, which may be utilized in unregulated quantities on a regular basis. The phytochemicals characterized as anti-*Salmonella* agents from edible plants should be superior to non-natural preservatives. In addition, alcohols have another superior property as antimicrobial agents compared with benzoic acid (**18**), a common commercial antimicrobial agent. As a weak acid antimicrobial agent, benzoic acid's activity is pH dependent and increases as the pH of the substrate decreases (Sofos, 1983). At higher pH values (>7) benzoic acid did not show any antibacterial activity against *S. choleraesuis* up to 1600 μg/ml, owing to a higher degree of dissociated molecules. In contrast, the alcohols are not affected by pH. This would seem to be of greater overall value than other pH-sensitive antimicrobials, because many foods have near neutral pH values. Moreover, alcohols were previously reported to inhibit soybean lipoxygenase-1 (Kuninori *et al.*, 1992).

16.4 Conclusion

In our previous studies on structure–antifungal activity relationships with a homologous series of acyclic 2*E*-alkenals, we reported the antifungal activity of amphipathic medium chain (C9–C12) 2*E*-alkenals against *S. cerevisiae*. This was largely due to their non-ionic surface-active properties, and the maximum activity can be obtained when balance between hydrophilic and hydrophobic portions becomes the most appropriate, possibly similar to that described for acyclic alkanols (Kubo *et al.*, 1995b, 2003). In other words, the antifungal activity of 2*E*-alkenals against *S. cerevisiae* is in part due to biophysical processes. This concept can be extended to the antibacterial activity of the same medium chain 2*E*-alkenals against *S. choleraesuis,* because in the time kill experiment: (i) lethality occurred notably quickly, within the first 1 h after the addition of 2*E*-dodecenal; (ii) bactericidal activity was found at any growth stage; and (iii) 2*E*-dodecenal rapidly killed *S. choleraesuis* cells in which cell division was inhibited by chloramphenicol. Moreover, the antimicrobial activity of 2*E*-alkenals is non-specific (Kubo *et al.*, 1995a; Bisignano *et al.*, 2001; Nakamura and Hatanaka, 2002; Lanciotti *et al.*, 2003) and the potency of the activity against *S. choleraesuis* was distinctly increased with each additional CH_2 group, up to 2*E*-dodecenal. The results observed support the ability of medium-chain 2*E*-alkenals to function at least in part as non-ionic surfactants. Similar relationships between the activity of antimicrobial compounds and their lipophilicity were previously observed with some membrane-active antimicrobial agents, such as alcohols and quaternary ammonium compounds (Lien *et al.*, 1968; Hamilton, 1971; Hansch and Clayton 1973; Daoud *et al.* 1983; Kubo *et al.*, 1993). For example, the antimicrobial activity of a series of alkyldimethylbenzylammonium chlorides was a parabolic function of their lipophilicity and maximized with alkyl chain lengths between C12 and C16 (Daoud *et al.*, 1983). The surfactant concept of the same series of aldehydes was well described (Lanciotti *et al.*, 2003).

Moreover, the leakage of carboxyfluorescein (CF) in liposomes of phosphatidylcholine (PC) following exposure to 2*E*-alkenals

was previously reported (Trombetta *et al.*, 2002), similar to those found for alkyl gallates (Fujita and Kubo, 2002). Interestingly, 2*E*-alkenals caused rapid CF leakage from PC liposomes, and the effectiveness order correlated well with the alkyl chain length. Thus, 2*E*-nonenal was more effective in inducing CF leakage from PC liposomes than was 2*E*-hexenal (Trombetta *et al.*, 2002). This previous report also supports the surfactant concept.

The activity often disappears after the chain length reached the maximum activity and this phenomenon is known as the cutoff (Balgavy and Devínsky, 1996). As expected, dodecanal (C12) was the most effective against *S. choleraesuis* with both MIC and MBC of 100 μg/ml, whereas tridecanal (C13) (**19**) did not show any activity up to 800 μg/ml. Noticeably, this cutoff was not observed with the 2*E*-alkenal series against *S. choleraesuis*. That is, 2*E*-tridecenal exhibits some activity, though to a lesser extent than 2*E*-dodecenal. This difference in susceptibility of *S. choleraesuis* to 2*E*-alkenals possessing different chain lengths still remains largely unclear. Because the hydrophobic forces are more favourable than hydrogen-bonding forces, this may help to explain the cutoff, in that the compound is pulled further into the membrane (Franks and Lieb, 1986) and loses the orientation required for bilayer disruption. Gram-negative bacteria including *Salmonella* sp. surround themselves with a double membrane. The inner or cytoplasmic membrane is mainly composed of phospholipids, whereas the outer membrane is an asymmetric structure containing primarily phospholipids in its inner monolayer and lipopolysaccharide in its outer monolayer (Snyder and McIntosh, 2000). The outer membrane acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Helander *et al.*, 1997). 2*E*-Alkenals at least overcome the outer membrane barrier through the following mechanism of disruption and access the cytoplasmic membrane. The hydrophilic aldehyde group first binds with an intermolecular hydrogen bond like a 'hook' by attaching itself to the hydrophilic portion of the cytoplasmic membrane surface, at which point the hydrophobic alkyl portion of the molecule is able to enter into the membrane lipid bilayers (Kubo *et al.*, 1995b).

The common nature among these aldehydes should be considered in that the electron negativity on the aldehyde oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be disturbed maximally by hydrophobic compounds of a particular hydrophilic aldehyde group. They could enter the molecular structure of the membrane with the polar aldehyde group oriented into the aqueous phase by hydrogen bonding and non-polar carbon chain aligned into the lipid phase by dispersion forces. Eventually, when the dispersion force becomes greater than the hydrogen-bonding force, the balance is destroyed and the activity disappears. In connection with this, the hydrophobic bonding energy between an average fatty acid ester and a completely hydrophobic peptide is approximately 12 kcal/mol. Addition of a hydrogen bond between a peptide and a fatty ester's carbonyl adds another 3–6 kcal/mol. Furthermore, aldehydes first approach the binding site with the electron negativity of the aldehyde oxygen atom. This hydrogen bond acceptor will affect the hydrogen bonds that regulate the permeability of the lipid bilayer.

The process by which 2*E*-alkenals reach the action sites in living microorganisms is usually neglected in the cell-free experiment but this must be taken into account in the current study. The inner and outer surfaces of the membrane are hydrophilic, whereas the interior is hydrophobic, so the increased lipophilicity of 2*E*-alkenals should affect their movement further into the membrane lipid bilayer portions. It should be logical to assume that most of the lipophilic 2*E*-alkenal molecules being dissolved in the medium are partially incorporated into the lipid bilayers (Franks and Lieb, 1986), in which they may react with biologically important substances. The amount of 2*E*-alkenals entering into the cytosol or lipid bilayer is dependent on the

length of the alkyl chain. Hence, the length of the alkyl chain is associated with eliciting activity to a large extent.

The knowledge obtained may provide a new insight into the bactericidal action of 2*E*-alkenals on a molecular basis, and allow a more rational and scientific approach to the design of efficient and safe anti-*Salmonella* agents. For example, a minute amount of the medium-chain 2*E*-alkenals, such as 2*E*-dodecenal and 2*E*-undecenal, can be added to the artificial fodder to control *Salmonella* bacteria. Thus, 2*E*-alkenals have potential as a crop preservative to inhibit or prevent the growth of *Salmonella* bacteria. It should be noted, however, that 2*E*-alkenals are highly reactive chemicals, which may limit their practical application.

Lastly, it may be worthwhile to consider the anti-*Salmonella* activity of rather common phytochemicals from an ecological point of view. For example, green leaves containing a variety of antibacterial agents against *Salmonella* bacteria, especially those known as green leaf alcohols and aldehydes (Hatanaka, 1993), very likely to have controlled *Salmonella* in nature when chickens were continuously fed green-leaf-based foods. In the Amazon basin, *Salmonella* contamination of pepper has been increasingly noted with increasing large-scale poultry farms. This may be caused by shifting their foods from plant-based natural foods to artificial fodders. Although it is not possible to affirm that membrane damage is the only cause of the lethal effect, it can be concluded that the medium-chain alkanols target the extracytoplasmic region as surfactants. This is highly desirable because they do not need to enter the cell, thus avoiding most cellular pump-based resistance mechanisms. In addition, primary alcohols are considered to be chemically stable, colourless, inexpensive, biodegradable and essentially nontoxic to humans. Therefore, a minute amount of the medium-chain alcohols can be added to the artificial fodders to control *Salmonella* bacteria. Thus, alcohols have potential as crop preservatives to inhibit or prevent the growth of *Salmonella* bacteria.

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17 Photoprotective Activity of some Mexican Plants

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17.1 Introduction

In the past few decades several human activities, such as the release into the environment of chlorofluorocarbons, have led to the diminution of the ozone layer. This has resulted in an increase in the amount of solar radiation reaching the earth's surface. Ultraviolet radiation (UVR) from the sun is divided into UVC (270–290 nm), UVB (290–320 nm) and UVA (320–400 nm). The amount of UVR that crosses the atmosphere and reaches the earth is approximately 6% of the sun's radiation. The radiation intensity depends on several factors such as altitude, latitude, season of the year, hour of the day, cloudiness and light dispersion (Goettsch *et al.*, 1998; De Gruijil, 1999).

The skin is the body organ most exposed to UVR. Several studies *in vitro* and *in vivo* have demonstrated that UVB causes damage to various molecules and cellular components, and produces alterations in cell functions. The DNA is the main target; its damage can lead to skin cancer development (Marrot and Meunier, 2008). The harmful effects of UVR in the skin can be divided into acute (sunburn or erythema, phototoxic reactions, photoallergic reactions and photosensitivity) and chronic (photo-ageing, skin cancer and immunosuppression) (Matsumura and Ananthaswamy, 2004; Adhami *et al.*, 2008).

Experimental and epidemiologic evidences have shown the direct correlation between exposure to UVB with squamous cell cancer (SCC), basal cell cancer (BCC) and melanoma (Black *et al.*, 1997; De Grujil, 1999; Armastrong and Kricker, 2001; Matsumura and Ananthaswamy, 2004). Incidence of skin cancer has increased so fast in the last few decades that it has been considered as the silent epidemic of the 20th century (Stratton *et al.*, 2000). Cutaneous overexposure to solar radiation, especially its UVR component, can cause several skin-related disorders. In Mexico, skin cancer ranks second in frequency among all malignancies, which constitutes 13.6% in the last report of the 'Registro Histopatológico de Neoplasias Malignas' (Secretaría de Salud, 2001). Non-melanoma

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skin cancers comprising BCC and SCC are the most frequently diagnosed cutaneous malignancies in the world now and their incidence is increasing. It is well known that they are associated with a low mortality rate, but they have a high cost in the health system. In addition, they produce a broad range of disfigurement if the lesions are located in the head or neck. Melanoma is related to the frequency of severe sunburn in childhood. Melanoma can be treated with surgery if it is detected in time; however, when it presents metastasis there is no cure (Stratton *et al.*, 2000).

The UVR generates reactive oxygen species (ROS) and induced oxidative stress in skin cells. Oxidative stress may cause damage at the cellular level, as well as at the molecular level, and this can result in cutaneous inflammation, lipid and protein oxidation, DNA damage, and activation or inactivation of certain enzymes such as catalase (CAT) and superoxide dismutase (SOD), all of which could potentially contribute to UVB-induced photodamage of the skin. It has been estimated that there are approximately 35 DNA adducts in the presence of ROS; one of the key markers of oxidative DNA damage is 8-hydroxy-2-deoxy-guanosine (8-OHdG), which induces the change of guanine to thymine (T→G) (Ichihashi *et al.*, 2003). ROS are intracellular mediators that are implicated in signal transduction. ROS generation leads to the expression of specific genes involved in the development of pathological conditions such as immunosuppression and all stages of photocarcinogenesis in skin. NF-kB is a ubiquitously expressed transcription factor that regulates genes involved in inflammation, immunity, cell-cycle progression and apoptosis. NF-kB activation is subject to redox regulation. The AP-1 transcription factor is a dimer composed of proteins from the Fos and Jun families. AP-1 activation, which is also redox regulated, principally leads to cell proliferation and transformation. In addition, NF-kB and AP-1 are activated by UVR, either independently or coordinately regulating the expression of several target genes whose protein products are molecular markers of processes such as inflammation, immunosuppression and tumour transformation, such as cyclooxygenase (COX), nitric oxide synthase (NOS) and ornithine decarboxylase (ODC) (Afaq *et al.*, 2005).

17.2 Photoprotection

Exposure to UVR has several adverse effects on the skin. Actions recommended to prevent overexposure to UVR include: avoiding exposure to the sun; wearing clothing that protects the neck, arms and legs; using hats and sunglasses; using sunscreens and blockers with a sun protection factor (SPF) of 15 or higher; teaching children to protect themselves from the sun; deliberately avoiding tanning in tanning beds; and examining the skin regularly to detect the presence of early cancers (Agarwal and Mukhtar, 1996; Kullavanijaya and Lim, 2005).

Sunscreens are chemicals that can absorb UV light efficiently, are applied topically and protect from the adverse effects of sunlight, mainly erythema. Most of the commercial formulations contain several active ingredients for a broad SPF of 280–400 nm (UVB and UVA). The application of sunscreen before exposure to UV radiation prevents sunburn, DNA damage and skin cancer (Pinnell, 2003). However, sunscreens can induce side effects such as irritation, allergy, phototoxic reactions, can affect the synthesis of vitamin D, generate ROS and act as photosensitizers. These primary prevention approaches have had limited success. Therefore, additional efforts are needed to prevent skin cancer (Kullavanijaya and Lim, 2005).

Several studies have evaluated the protective effect of natural products against damage induced by UVR in cells, tissues, animals and humans. Photochemoprevention is the use of synthetic or natural substances that can prevent, delay or reverse the damage caused by UVR (Agarwal and Mukhtar, 1996; Surh, 1999; Straton *et al.*, 2000; Afaq *et al.*, 2005). The photochemoprevention involves substances capable of absorbing the UV and acting as filters, preventing DNA damage and immunosuppression. In addition,

polyphenolic compounds with antioxidant properties may prevent inactivation of enzymes such as CAT and SOD as well as prevent photo-oxidative damage to DNA, proteins and lipids. On the other hand, exposure to UVR initiates a cascade of events that alters gene expression and thus the signal transduction involved in inflammation, apoptosis, immunosuppression and cell proliferation (Afaq *et al.*, 2005).

In recent years epidemiological and experimental studies have focused on a wide variety of natural products that provide protection to the development of skin cancer because they can alter or correct a variety of cellular functions induced by the UVR. The use of natural products as photochemopreventive agents can contribute in reducing the risk of skin cancer in combination with changes in lifestyle, diet and products for skin care. Within this concept, a variety of polyphenolic compounds with antioxidant, photoprotective, anti-inflammatory, immunomodulatory and antimutagenic properties has been reported (Afaq *et al.*, 2005; Adhami *et al.*, 2008).

Currently, in our laboratory we are interested in contributing to the phytochemical study of various Mexican species, as well as providing basic knowledge about the ability of secondary metabolites to prevent short-term UVB-radiation-mediated damage in skin. These compounds possess sunscreen properties and antioxidant activity, which can prevent the formation of erythema and avoid a state of oxidative stress in the skin exposed to UVR. In addition, phytochemicals may have the advantage of being biodegradable and will probably present fewer side effects than the synthetic sunscreens. This knowledge provides a guideline for future studies that will determine whether these metabolites are involved in other processes such as inflammation and immunosuppression, as well as understanding the molecular mechanisms of photochemopreventive agents.

In this chapter we present a review of the antioxidant and photoprotective potential of the extracts and metabolites isolated from various Mexican plants. Some flavonoids, stilbenes and phenylpropanoids were isolated from two species of *Buddleja* and *Yucca periculosa*. The natural compounds and some derivatives have antioxidant activity. In addition, the photoprotective effect of substances against UVB-induced cellular death was evaluated through challenge experiments using *Escherichia coli*. Photoprotective activity was also evaluated in an animal model. The SPF of the substances was evaluated by a guinea pig bioassay and a histopathological skin study.

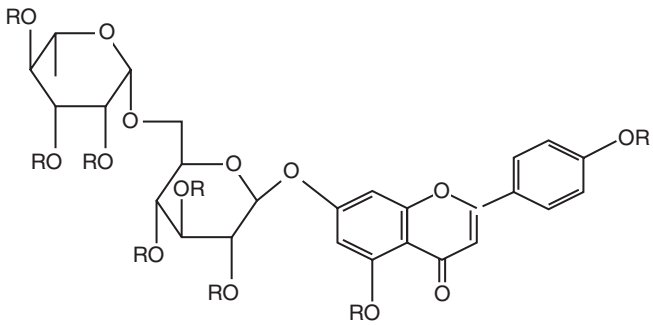
17.3 Natural Products Isolated from *Buddleja*

The *Buddleja* genus (Buddlejaceae) comprises about 100 species, 50 of which are native to the Americas. *Buddleja* species are small trees or shrubs that grow in tropical lands of America, Asia and Africa. In Mexico there are 20 species of *Buddleja*, which are distributed throughout the national territory (Rzedowski and Rzedowski, 1985). Some of these plants are used by Mexican traditional medicine for the treatment of erysipelas, such as healing, analgaesic, antidiarrhoea, anti-inflammatory and infections of the upper respiratory tract (INI, 1994). *Buddleja perfoliata* HBK is distributed in the states of San Luis Potosí, Querétaro, Hidalgo and Puebla in Mexico, is known popularly as 'salvia real' or 'salvia de bolita' and is used as an antiperspirant, a diuretic, to disinfect wounds and for gastrointestinal infections. This plant is a shrub that can reach 1 m in height, is branched with dense pubescence and has leaves that are lanceolate oblong, with finely crenate margins, with venation prominent on the underside. *Buddleja scordioides* HBK is a shrub that grows in the Chihuahuan desert (Rzedowski and Rzedowski, 1985). Decoctions of this plant are used orally or topically for treatment of several illnesses such as diarrhoea, headache and pain (Aguilar *et al.*, 1994). In the surroundings of Dr Arroyo, Nuevo León México, where *B. scordioides* is known as 'escobilla', the outdoor workers use an infusion of its aerial parts as a sunscreen (personal communication).

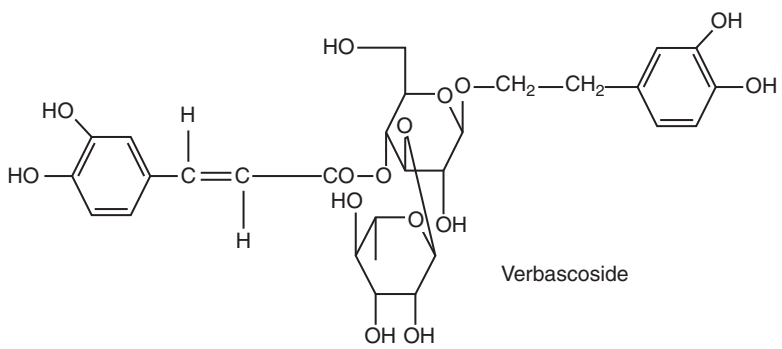
B. perfoliata and *B. scordioides* contain linarin and verbascoside (Fig. 17.1) (Avila, 2002; Avila *et al.*, 2005). Linarin is a flavonoid (acacetin-7-O-beta-d-rutinoside); it showed a selective dose-dependent inhibitory effect on acetylcholinesterase (Oinonen *et al.*, 2006). Linarin is also reported to induce apoptosis in human prostate cancer cells, which was also accompanied by poly (ADP-ribose) polymerase (PARP) cleavage (Singh *et al.*, 2005), has sedative and sleep-enhancing properties in mice (Fernández *et al.*, 2004), and activate macrophages and modulate cytokine production (Han *et al.*, 2002). It also possesses anti-inflammatory, analgaesic, diaphoretic, hypotensive, anti-stress/anxiety, antipyretic (Martínez-Vázquez *et al.*, 1998) and amoebicidal activities (Rodríguez-Zaragoza *et al.*, 1999). We isolated and also prepared linarin peracetate to evaluate the photoprotective properties. Verbascoside or acteoside is an active phenylpropanoid glycoside. This compound is structurally characterized by caffeic acid and 4,5-hydroxyphenylethanol bound to a β-(D)-glucopyranoside, found in bitter tea and many medicinal plants, has exhibited a wide biological activity, free-radical scavenging being the most representative one. It is a powerful antioxidant either by direct scavenging of reactive oxygen and nitrogen species or by acting as chain-breaking peroxyl radical scavenger (Korkina, 2007; Perron and Brumaghim, 2009). Moreover, anti-tumour, antimicrobial (Avila *et al.*, 1999), anti-inflammatory (Speranza *et al.*, 2010), analgaesic, antipyretic (Wahba *et al.*, 2010), anti-thrombotic and wound healing (Korkina *et al.,* 2007), and chemopreventive (Hwang *et al.*, 2010) properties have been previously described.

17.4 Natural Products Isolated from *Yucca periculosa*

The genus *Yucca* has about 35 to 40 species, which makes it the second largest genus of



Linarin: R = H; Linarin acetate: R = Ac



Verbascoside

Fig. 17.1. Structures of UVB-absorbing compounds isolated from *Buddleja perfoliata* and *Buddleja scordioides*.

the family Agavaceae. These are perennial, succulent plants (Pellmyr *et al.*, 2007). *Yucca periculosa* is an endemic plant in the Mexican states of Puebla, Oaxaca, Tlaxcala and Veracruz. It is known as 'ntaxha'o', 'izote' or 'palmillo'. It is a perennial tree, a succulent measuring 5–15 m tall; its trunk is generally smooth, although there may be ramifications. These plants have rigid leaves that are concave, glabrous, and linear-lanceolate with a finely fibrous margin and acute apex ending in a spine; the leaves are clustered toward the ends of the branches (Matuda and Piña, 1980).

Y. periculosa has several phenolic compounds with antioxidant properties (Fig. 17.2). Resveratrol and *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (MS) have previous been isolated from the methanolic extract of the bark of this plant (Torres *et al.*, 2003). MS showed strong radical scavenging and even stronger anti-platelet activity than did resveratrol (Piacente *et al.*, 2004). This compound, like resveratrol, has protective effects against peroxynitrite-induced oxidative/nitrative damage to human platelet proteins and lipids (Olas *et al.*, 2008). Resveratrol (3,4′,5-trihydroxystilbene) has been found in various plants, including grapes, berries and peanuts (Dong, 2003). In recent years it has been the focus of many studies aimed at understanding its full range of beneficial health effects (De la Lastra and Villegas 2007; Pezzuto, 2008), which include mainly antioxidant and anti-inflammatory activities, anti-platelet aggregation effects, anti-atherogenic properties, oestrogen-like growth-promoting effects, growth-inhibiting activities, immunomodulation and chemoprevention. We also isolated from *Y. periculosa* a flavanone, naringenin (García-Bores *et al.*, 2010). This compound possesses some antioxidant activity, but its activity is poor in comparison with many other polyphenols and flavonoids (Erlund, 2004).

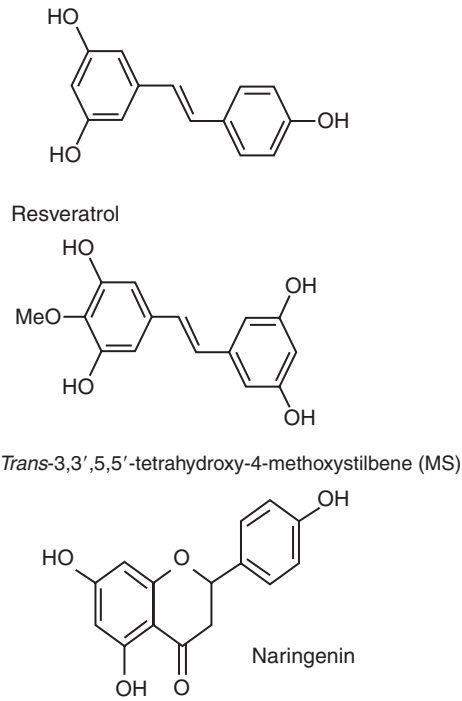


Fig. 17.2. Structures of UVB-absorbing compounds isolated from *Yucca periculosa.*

17.5 Molar Extinction Coefficients

One of the features of photochemopreventive agents is the ability to absorb UVR. Because of this, the first step in our research is to determine whether extracts and isolated metabolites absorb UVR. The UVB-absorbing compounds linarin and verbascoside were isolated from the methanolic extract of *B. perfoliata* and *B. scordioides*, and linarin acetate was prepared in our laboratory. Resveratrol, MS and naringenin were isolated from the methanolic extract of *Y. periculosa.* We have reported the λ_{max} and molar extinction coefficient of natural compounds isolated from *B. perfoliata* (Avila, 2002)*, B. scordioides* (Avila, 2002; Avila *et al.*, 2005) and *Y. periculosa* (García-Bores *et al.*, 2010).

As shown in Table 17.1, the methanolic extracts and compounds isolated have the maximum absorption in the UVB region of the electromagnetic spectrum and are therefore potentially photoprotective substances. In addition, the metabolites have similar extinction coefficients to those reported for photoprotective substances used commercially (Shaath, 1997, 2005; Pattanaargson *et al.*, 2004).

Table 17.1. Absorption in the UVB range of the methanolic extracts and molar extinction coefficient of the compounds from *B. perfoliata, B. scordioides* and *Y. periculosa*, and several commercial sunscreens.

Substance	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)
Methanolic extract *B. perfoliata*	282, 320	n.d.
Methanolic extract *B. scordioides*	279, 316	n.d.
Linarin	334	18,832
Linarin acetate	320	32,335
Verbascoside	291, 332	26,130, 35,113
Methanolic extract *Y. periculosa*	232, 313	n.d.
Naringenin	212, 288	12,555, 7,745
Resveratrol	218, 305	29,823, 37,895
trans-3,3′,5,5′-tetrahydroxy-4-methoxystilbene (MS)	227, 316	40,278, 45,374
Commercial sunscreens		
Octyl-*p*-methoxy-*trans*-cinnamate (OMC) (Pattanaargson *et al*., 2004)	310	24,000
Octylsalicylate (Shaath, 2005)	308	4,900
Avobenzone (Shaath, 2005)	351	30,500
p-aminobenzoic acid (Shaath, 2005)	293	14,000
Padimate-O (Shaath, 2005)	307	27,300

n.d., not determined.

17.6 Photoprotective Effect Against UVB-induced Cell Death

The protective effect against UVB-induced cell death was evaluated using *E. coli* as a cell model. The bacterial decay depends mainly on the dose of radiation that induces damage to DNA. *E. coli* was inactivated when exposed to UV. The effectiveness of UV light in the biological inactivation primarily results from the fact that DNA molecules absorb UV photons between 200 and 320 nm, with peak absorption at 265nm. In case of lethal damage, DNA replication is blocked by DNA alterations, mainly cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6–4) pyrimidinone (6–4PP), which ultimately results in reproductive cell death. The exposure of a bacterial culture to UVB produces the rapid decline in population caused by damage to the DNA (Oguma *et al.*, 2001; Taghipour, 2004).

Our results showed that the bacteria population (≈10^8) without protection reached cell death at 10 min, with a mortality rate (K) of 0.8276 (Fig. 17.3a) (García-Bores *et al.,* 2010). Naringenin possesses pronounced photoprotective activity when compared with the negative control; although the results show that it was less active than octyl-*p*-methoxy-trans-cinnamate (OMC) as a protective control (cell death at 35 min). Methanol extracts of the Mexican plants studied (Fig. 17.3b) and resveratrol protected their respective bacteria populations in a similar manner to OMC and did not reach cell death until 60 min. Linarin and MS protect against cell death in bacterial population to 120 min. Linarin acetate and verbascoside protected the bacteria more efficiently than the positive control; the bacterial population protected by those compounds did not reach cell death until 120 min of irradiation with UVB (Fig. 17.3c).

The constant mortality K is a parameter that indicates the range of inactivation of *E. coli*. The data in Fig. 17.3b and 17.3c show the photoprotective effect of the methanolic extracts of *B. perfoliata, B. scordioides* and *Y. periculosa* tested. All substances (OMC, methanolic extracts, linarin, linarin acetate, verbascoside, naringenin, resveratrol and MS) protected the bacterial population from the lethal effects of UVR. All substances presented K values lower than experiments without protection (Table 17.2). In the experiments with protection, the K ranged from 0.03 to 0.27. Verbascoside and linarin acetate showed a strong photoprotective

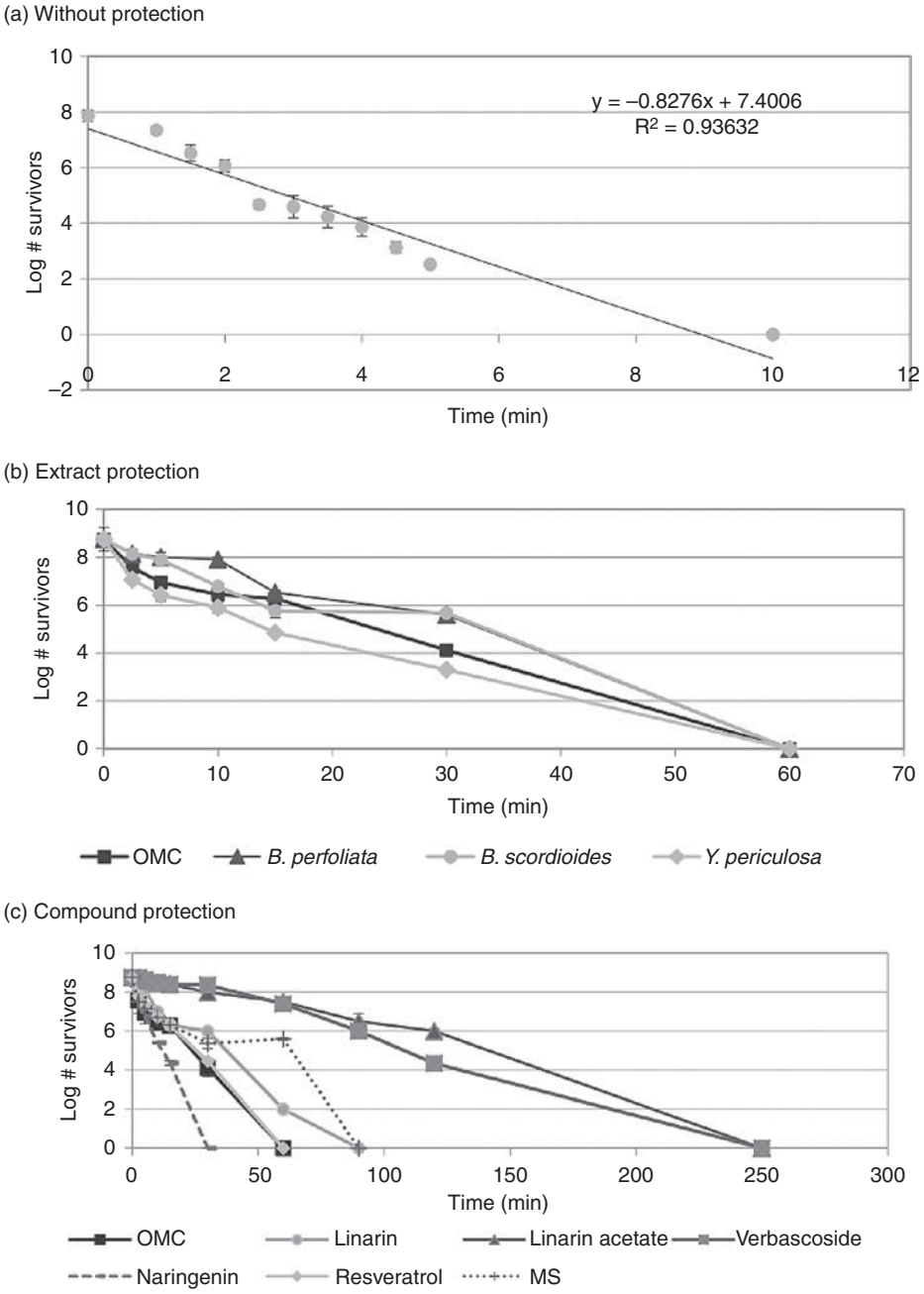


Fig. 17.3. Protective effect against UVB-induced cell death of *E. coli*. (a) Without protection (K = 0.82, R^2 = 0.93). (b) Extracts and OMC protection. (c) Compound protection. MS: *trans*-3,3′,5,5′-tetrahydroxy-4-methoxystilbene; OMC: Octyl-*p*-methoxy-*trans*-cinnamate.

Table 17.2. Coefficient of determination (R^2) and mortality constant (K) of bacterial irradiation experiments with and without protection.

Substance	K	R^2	Protection of cell death (min)
Without protection	0.8276	0.9363	5
OMC	0.1349	0.9832	30
Methanolic extract *B. perfoliata*	0.1404	0.9723	30
Methanolic extract *B. scordioides*	0.1357	0.9547	30
Linarin	0.0971	0.9790	60
Linarin acetate	0.0323	0.9623	120
Verbascoside	0.0347	0.9912	120
Methanolic extract *Y. periculosa*	0.1306	0.9482	30
Naringenin	0.2766	0.9911	15
Resveratrol	0.1372	0.9915	30
MS	0.0746	0.8549	60

MS: *trans*-3, 3′, 5, 5′-tetrahydroxy-4-methoxystilbene; OMC: Octyl-*p*-methoxy-*trans*-cinnamate

effect against UVB-induced cell death; the K (0.03) was 27.5-fold below the K without protection (0.82). Our research shows the potential photoprotective effect of extracts and compounds isolated from Mexican plants.

17.7 Photoprotective Activity Against UVB-induced Skin Damage: Sun Protection Factor (SPF)

Sunburn erythema is the most conspicuous and well-recognized acute cutaneous response to UV irradiation, and it is the most widely used end point in dermatological photobiology. The molecules responsible for light absorption (chromophores) that initiate sunburn inflammation have not been precisely identified. However, the action spectrum of erythema is consistent with the hypothesis that UV interactions with DNA are of major importance. Indirect oxidative damage might also occur secondarily to endogenous photosensitization reactions (Matsumura and Ananthaswamy, 2004).

A widely accepted method for sunscreen efficacy measurements is SPF, which is defined as the ratio of the dose of UVR (290–400 nm) required to produce 1 Minimal Erythema Dose (MED) on sunscreen-protected skin (after application of 2 mg/cm^2 of product) over the dose required to produce 1 MED on unprotected skin (Bissett *et al.*, 1991). Sunscreens have long been used to protect against the acute effects of UVR. OMC is a widely used UVB filter in various cosmetic formulations. It is known that all organic sunscreen agents may induce adverse effects such as irritation, allergic contact reaction, photoallergy or phototoxicity. Kullavanijaya and Lim (2005) reported photosensitization and/or photoallergic reactions induced by this compound. Because of this, it is important to develop research focused on the search for natural substances with photoprotective activity.

The SPF of all substances was determined on guinea pigs in our laboratory (Table 17.3). The negative control (guinea pigs with vehicle) showed perceptible erythema at 20 ± 2 min; this time was considered as the MED. All the substances were more active than OMC (SPF 2.0 ± 0.1), because a significant difference was observed in comparison with controls. The SPFs obtained from plant extracts and naringenin were ≅3, and from linarin acetate, resveratrol and MS were ≅5. Linarin and verbascoside were the compounds with the highest photoprotective activity: their SPFs were 9 and 24, respectively (Avila *et al.*, 2005; García-Bores *et al.*, 2010). Verbascoside retarded the appearance of erythema at about ~440 min. The methanolic extracts and the phytochemicals have maximum absorptions in the UVB region of the electromagnetic spectrum and will be, therefore, potentially photoprotective substances (Table 17.1). This explains the protective properties from these Mexican plants.

Table 17.3. Sun protection factor (SPF) of the substances evaluated in guinea pigs.

Compounds (2 mg/cm^2)	SPF	Exposition time without erythema (min)
Without protection	–	20 ± 2.0
OMC	2.0 ± 0.10	40 ± 4.5
MeOH extract of *B. perfoliata*	3.0 ± 0.09	60 ± 1.8
MeOH extract of *B. scordioides*	3.0 ± 0.09	60 ± 1.8
Linarin	9.0 ± 0.30	160 ± 6.0
Linarin acetate	5.0 ± 0.2	80 ± 4.0
Verbascoside	24.0 ± 0.7	440 ± 14
MeOH extract of *Y. periculosa*	3.4 ± 0.5	68 ± 9.5
Naringenin	3.6 ± 0.6	72 ± 10.1
Resveratrol	5.0 ± 0.7	100 ± 12.3
MS	5.6 ± 0.5	112 ± 8.5

MS: *trans*-3,3′,5,5′-tetrahydroxy-4-methoxystilbene; OMC: Octyl-*p*-methoxy-*trans*-cinnamate.

17.8 Photoprotective Activity Against UVB-induced Skin Damage: Histological Study

A histological evaluation was also performed on the guinea pig skin exposed to UVR, both the unprotected skin and the skin protected by each of the substances. The histological changes after 20 min of UV irradiation in guinea pig skin compared with normal skin (Fig. 17.4a) include thickening of stratum corneum and epidermis, intra/intercellular and perivascular oedema, perivascular infiltration and spongiosis, as shown in Fig. 17.4b. The guinea pigs treated with a topical, sub-minimal erythema dose of the methanolic extracts, the isolated compounds or OMC did not show these UVB-induced inflammatory changes, as shown in Fig. 17.4c–17.4l. The histopathological study of the skin samples exposed at MED with protection showed that a topical application of each of the experimental treatments had a different effect on the skin, which could be an indication that the protection afforded was also linked to the modulation of cellular processes. The appearance of erythema in animals treated with resveratrol, linarin, linarin acetate and MS occurred at ~100 min, while those animals treated with naringenin or the methanol extracts of plants had an appearance of erythema at ~70 min, and with OMC had an appearance of erythema after ~40 min of exposure to UVR. Finally, those animals treated with verbascoside retarded the appearance of erythema at about ~440 min.

Many agents, like UV light filters, affect the transmission of UV light to human skin. In addition, there are agents like antioxidants that can modulate the effects of UV light on the skin. Most of the naturally occurring chemopreventive phenolics exert multifaceted action, and any clinical applications using these substances should be based on the precise understanding of the physiologically relevant action mechanisms.

17.9 Conclusion

The increase in skin cancer morbidity and mortality is alarming and expensive, in both human and economic terms. New strategies are needed to combat this disease. The development of promising chemopreventive agents is a demanding process that requires continuous research. The natural products constitute an important group of pharmacological agents capable of preventing the occurrence and reducing the severity of UVR-induced skin diseases. The research of natural products with chemopreventive properties has focused on the antioxidant, anti-inflammatory and antimutagenic activities of the compounds. In addition, this chapter shows that the phenolic compounds isolated from *B. perfoliata,*

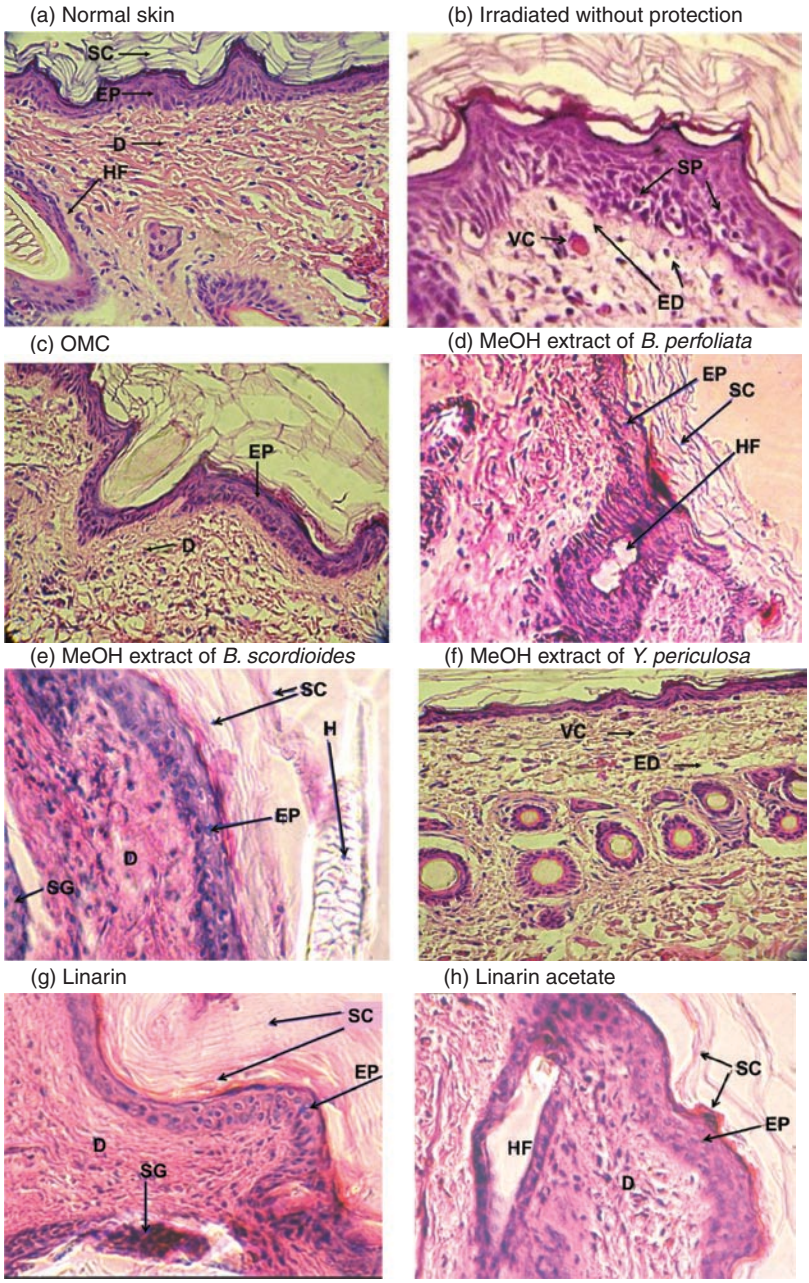


Fig. 17.4. Histology of skin of UVB-irradiated guinea pig treated with photoprotective substances from Mexican plants, ×20. (a) Normal skin, (b) Irradiated without protection, (c) OMC, (d) MeOH extract of *B. perfoliata*, (e) MeOH extract of *B. scordioides*, (f) MeOH extract of *Y. periculosa*, (g) Linarin, (h) Linarin acetate, (i) Verbascoside, (j) Naringenin, (k) Resveratrol,(l) MS. D: dermis, ED: edema, EP: epidermis, H: hair, HF: hair follicle, SC: stratum corneum, SG: sebaceous gland, SP: spongiosis, VC: vassel congestion.

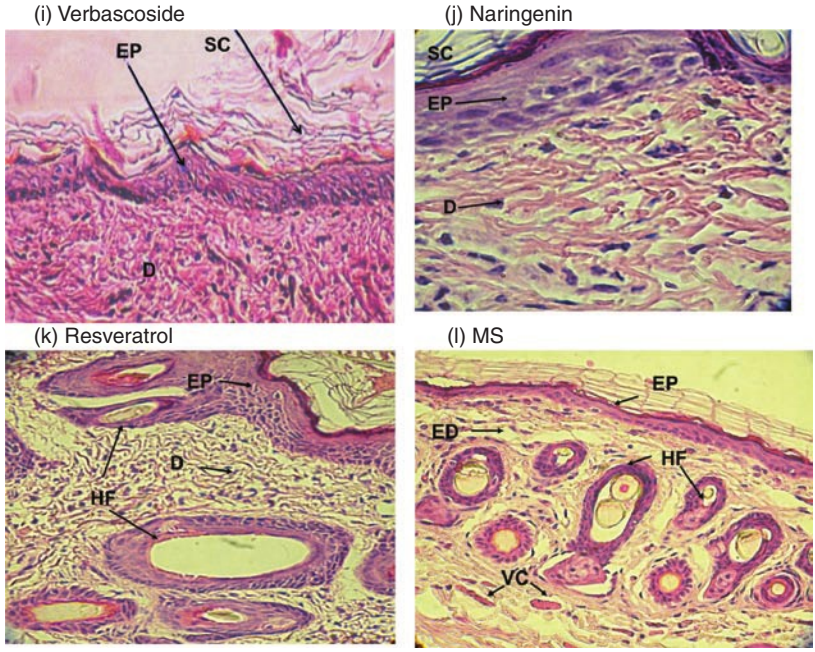


Fig. 17.4. Continued.

B. scordioides and *Y. periculosa* are able to absorb UVR, reducing the transmission of this type of radiation to the skin. These compounds thus provide photoprotection because of their antioxidant properties and act as a sunscreen.

Acknowledgements

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